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Chemical Properties of Two Mucoids from Bovine Cervical Mucin

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Our understanding of the chemistry and physical chemistry of epithelial mucin has in the past been handicapped by the lack of methods of fractionation which did not, at the same time, destroy the essential physical properties of the original secretion. Earlier work has, in the main, been confined to the analysis of the crude material (Boyland, 1946; Folkes, Grant & Jones, 1950; Bergman & Werner, 1951; Werner, 1952; Bacila & Ronkin, 1952), or, where purification has been carried out, no effort has been made to preserve the viscoelastic gel-like properties of the mucin. In the latter case, a particular biological activity has usually been the object of the purification procedure, e.g. virulence-enhancing activity (Smith, 1951; Smith, Gallop & Stanley, 1952) or blood-group serological activity (see Kabat, 1956). Indeed a primary object in purification has usually been the destruction of the physical properties of the mucin to facilitate fractionation. To this end, enzymic digestion (Kabat, 1956; Lawton, McLoughlin & Morgan, 1956) and alkali-treatment (Satoh, 1949; Yosizawa, 1950) have frequently been employed. An alternative approach has been the use, as starting material, of a secretion in which a substantial proportion of the mucoid is readily soluble, e.g. urine (Freudenberg & Molter, 1939), ovarian-cyst fluid (Gibbons, Morgan & Gibbons, 1955) and meconium (Rappoport & Buchanan, 1950). A recent review (Bettleheim-Jevons, 1958) summarizes the present state of our knowledge of the chemistry of these substances.

The object of this work was to obtain the constituent responsible for the physical properties of native mucin as an essentially homogeneous preparation in a condition as near to the native state as possible in order to study the factors which underlie these physical properties. Bovine cervical mucin has been used as source material since this secretion shows marked physical changes during the oestrous cycle (Scott-Blair, Folley, Malpress & Coppen, 1941). Both the thick plasto-elastic gel secreted during pregnancy and the thinner viscoelastic 'spinnbar' material secreted at oestrus can be conveniently obtained and studied. A study of this secretion may throw some light on the mechanism of fertilization in mammals; furthermore, it is reasonable to suppose that the physical properties of the secretion are controlled by the circulating sex hormones. There is already some experimental evidence in support of this expectation (F. A. Glover, unpublished observations), so that this study may also have some interest for the endocrinologist. The secretion appears to be a fairly simple one, containing little extraneous material other than protein, and although some contamination with urinary and faecal matter is inevitable, this can, with care, be relatively slight. In this report the chemical properties of two mucoids, isolated from oestrous mucin and pregnancy mucin respectively, are described. The physicochemical properties of these preparations are described in the next paper (Gibbons & Glover, 1959).

EXPERIMENTAL

Materials and methods

Fractionation. The figures quoted for g refer to the force exerted at the centre of the centrifuge tube.

Mucin from the external os of the uterus of the cow was collected and stored frozen until used. The mucin from a number of cows was pooled, but pregnancy and oestrus samples were kept separate. Oestrous mucin was thawed at room temperature and centrifuged at 44 000 g at 0° for 90 min. in a Spinco preparative ultracentrifuge; the aqueous phase was removed and the gel phase allowed to stand with an equal volume of water with occasional gentle shaking for 12–24 hr. at 4°. The centrifuging was then repeated and the gel phase again allowed to stand with water. This procedure was repeated once more, the supernatants being pooled. The gel phase was allowed to stand with five times its volume of aqueous saturated $CaCl_2$ soln.–ethanol (9:1, v/v). This solvent has been found more effective than saturated $CaCl_2$ alone. The mucin usually dissolves in 1–2 days at 4°, but it is sometimes necessary to bring the mucin to room temperature to effect solution. Sufficient ethanol is then added at 4° to make the solution 20% (v/v) with respect to ethanol, when solid debris may be removed by centrifuging at about 2000 g. Further ethanol is then added at 4° with gentle agitation by hand, to 50% (v/v), when a fraction is removed by sedimentation at 44 000 g. Further fractions may be recovered between 50 and 60% (v/v) of ethanol, and between 60 and 90% (v/v) of ethanol. The first fraction is again dissolved in saturated $CaCl_2$ soln.–ethanol (9:1, v/v) and reprecipitated at 50% (v/v) of ethanol; this procedure is repeated once more if the addition of excess of ethanol to the supernatant produces anything more than a slight opalescence. Table 1 shows the analysis of fractions separated during this procedure.

Pregnancy mucin was allowed to stand with ten times its volume of water before the initial centrifuging, when considerable swelling usually occurs. Subsequently the material was worked up in exactly the same way as the oestrus material. The fractions precipitated below 50% ethanol concentration from both materials were found to sediment with a single boundary in the ultracentrifuge and were considered worthy of detailed study. Their chemical properties are described herein and their physical properties in the next paper (Gibbons & Glover, 1959), where the ultracentrifuge schlieren diagrams are given (Fig. 8). They were either dissolved in saturated $CaCl_2$ soln.–ethanol (9:1, v/v) and kept at $-10°$ or dialysed free of Cl ion at 4° (when the solution becomes a gel) and stored frozen until required. Samples required for ash determinations were dialysed free of Cl ion, then dialysed for 48 hr. against 0·5% sodium ethylenediaminetetra-acetate solution which had been brought to pH 6·0 (indicator paper) by addition of 3 N-NaOH, and finally dialysed against several changes of distilled water.

Materials in the supernatants obtained during preparative ultracentrifuging of the mucin could be further split into two fractions by centrifuging at 103 000 *g* for 5 hr. (see Table 1).

Fractionation for sialoprotein. The following experiments were performed in order to detect the presence of a possible sialoprotein component similar to that isolated from salivary mucin by McCrea (1953) and Heimer & Meyer (1956). (i) Oestrous mucin (80 ml.) was made 6 M with respect to urea and further treated as described by Heimer (1957). (ii) A specimen of the supernatant after ultracentrifuging of the crude oestrous mucin (fraction 2, Table 1) was fractionated with ethanol in the presence of Ba^{2+} ions, as described by Curtain & Pye (1955). (iii) A specimen of freeze-dried oestrous mucin (1 g.) was extracted with phenol–water (9:1, v/v) (Morgan & King, 1943) and the insoluble portion again extracted with phenol–ethylene glycol (7:3, v/v) (Annison & Morgan, 1952*a*). (iv) A further specimen (1 g.) of dried oestrous mucin was treated with ficin for 16 hr. at 37° in the presence of toluene (Lawton *et al.* 1956) and the digest after freeze-drying was extracted with phenol–water (9:1, v/v).

Mild acid hydrolysis. A sample of mucoid prepared from oestrous mucin (about 50 mg.) was allowed to stand at room temperature for 48 hr. in 10 ml. of 0·1 N-HCl. This solution was then fractionated by addition of ethanol, material being removed at 60 and 80% (v/v) of ethanol. The final supernatant was neutralized with 1 ml. of N-NaOH, evaporated at reduced pressure to small volume and freeze-dried. Material for chromatography was extracted with ethanol from the dried solids.

Analytical methods

Solutions for analysis were prepared by dissolving the dried material in 0·5 N-H_2SO_4 or 0·5 N-HCl; where this was undesirable a portion of the gel was dissolved in a little water with a few drops of N-NaOH and subsequently neutralized with N-H_2SO_4. In the latter case the concentration of the solution was determined by estimating the nitrogen separately on a dried sample of the same fraction. Aqueous solutions of the untreated mucoids are unsuitable for volumetric analysis.

Samples were prepared for reducing-sugar and hexosamine analyses by hydrolysis in a sealed Pyrex test tube for 16 hr. in 0·5 N-HCl at 100°; under these conditions hydrolysis is complete, and destruction of monosaccharides, other than sialic acid, is negligible (see Fig. 1). A portion of the hydrolysate (1 ml.) was then diluted, neutralized with

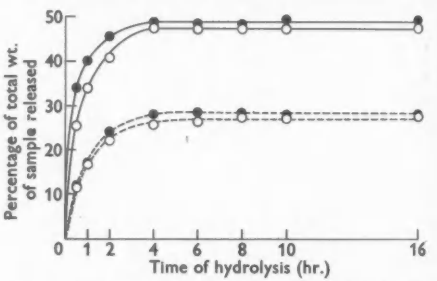

Fig. 1. Rate of hydrolysis of oestrous mucoid (○) and pregnancy mucoid (●) in 0·5 N-HCl at 100°. - - - -, Hexosamine released (as glucosamine base); —, reducing sugars released (as glucose).

Table 1. *Analysis of materials obtained during fractionation of bovine cervical mucin*

Fraction	Mucin	N* (%)	Hexosamine† (%)	Reducing sugar‡ (%)	Galactose (%)	Fucose (%)	Sialic acid§ (%)
1. Material not sedimenting at 103 000 *g*, 5 hr.	Oestrus	12·6	7·5	16·0	13·8	1·2	6·5
	Pregnancy	13·5	11·4	16·0	14·1	1·0	7·7
2. Material sedimenting at 103 000 *g*, 5 hr., but not sedimenting at 44 000 *g*, 1½ hr.	Oestrus	8·5	16·7	31·5	21·4	3·8	11·0
	Pregnancy	7·4	15·1	33·1	20·2	4·1	12·4
3. Material sedimenting at 44 000 *g*, 1½ hr., precipitated from aq. sat. $CaCl_2$ between 50 and 60% of ethanol	Oestrus	6·5	24·7	45·0	28·0	5·0	13·4
	Pregnancy	6·0	26·8	48·8	29·6	5·0	15·3
4. As 3, material precipitated above 60% (v/v) of ethanol	Oestrus	10·2	5·1	11·2	7·5	0·8	7·3
	Pregnancy	8·9	16·4	29·5	18·9	2·0	9·8

* Kjeldahl. † As base. ‡ As glucose. § As the *N*-acetylated compound.

0·5 ml. of N-NaOH and made up to known volume (usually 10 ml.). For paper chromatography, the hydrolysate was passed down a small (0·5 cm. × 8 cm.) column of Amberlite IR 4B (British Drug Houses Ltd.) in its acetate form, washed through with about 10 vol. of water and the effluent freeze-dried. For amino acid analysis and paper chromatography a similar hydrolysate in 6N-HCl was prepared, which was taken to dryness *in vacuo* over silica gel and solid NaOH. Other estimations were made on the intact materials.

Nitrogen. Kjeldahl nitrogen was estimated in the apparatus of Markham (1942).

Fucose. The method of Gibbons (1955) was used; with the low fucose values encountered, interference from galactose was serious; this was allowed for by making up standards containing appropriate amounts of galactose.

Hexosamine. The method given by Rondle & Morgan (1955) was followed. Results are expressed in terms of glucosamine base.

Reducing sugars. The colorimetric technique of Nelson (1944) was used. Results are expressed in terms of glucose.

Galactose. The anthrone method described by Yemm & Willis (1954) was employed, the extinction at 625 mμ being corrected for the absorption due to the fucose content. This is not a wholly valid procedure since fucose and galactose interact on heating in strong H_2SO_4, but in view of the small amounts of fucose present the error involved is likely to be within experimental error. Sialic acid gives no colour with anthrone.

Sialic acid. The acid *p*-dimethylaminobenzaldehyde reagent of Werner & Odin (1952) was employed. Authentic *o*-sialic acid (*N*-acetylneuraminic acid) was used as standard, in terms of which sialic acid analyses are given.

'N-Acetylhexosamine' colour. The *N*-acetylhexosamine colour–time of heating relationship was obtained under the standard conditions recommended by Aminoff, Morgan & Watkins (1952).

Acetyl group. A weighed sample (20–25 mg.) in 4–5 ml. of N-H_2SO_4 was hydrolysed in a sealed ampoule at 100° for 16 hr. When cool, the contents and washings were transferred quantitatively to a Markham still and 10 ml. of conc. H_2SO_4 was added. Volatile acid was then steam-distilled for 3 min. into 3 ml. of boiled water. The distillate was titrated with 11·7 mN-$Ba(OH)_2$ solution with methyl red–bromothymol blue indicator. *N*-Acetylglucosamine and acetanilide both yield acetic acid quantitatively under these conditions.

Amino acid analysis. The amino acids in hydrolysates (6N-HCl; 16 hr. at 100°) of both mucoids were separated and estimated on two resin columns (Moore & Stein, 1951, 1954). The peak effluent volumes were used to identify the amino acids present by comparison with analyses of known mixtures. Confirmation by paper chromatography was also obtained. Under the conditions used the two hexosamines emerge between phenylalanine and lysine as a single very large component. The absence of hydroxyproline was confirmed by using the specific reaction of Newman & Logan (1950) on tubes in the appropriate region.

Hexosamine ratios. The ratio of glucosamine: galactosamine was determined on the same hydrolysates as the amino acids by the method of Gardell (1953) as modified by Crumpton (1958).

Lysozyme. Activity was estimated approximately by observing the initial rate at which turbidity decreased, with a standard suspension of *Micrococcus lysodeikticus* in 0·066M-phosphate buffer, pH 6·8. A photoelectric colorimeter (Morris, 1944) with a red filter was employed.

Ash. The residue remaining after igniting 100–150 mg. at 500° for 5 hr. in an electric furnace was weighed. Owing to shortage of material, these estimations were not duplicated.

Lipids. These were determined by extraction of the dried material (about 50 mg.) with ether in a Soxhlet apparatus, the soluble material being subsequently estimated as described by Paul (1958) with tristearin as standard. Chloroform after ether did not extract appreciable amounts of additional material.

Paper chromatography. The usual one-dimensional descending technique was employed with the following solvents and spray reagents.

(i) Sugars and amino sugars (0·5N-HCl hydrolysates): solvent 1. Ethyl acetate–pyridine–acetic acid–water (Fischer & Nebel, 1955); solvent 2. Butanol–acetic acid–water (Partridge, 1948); spray reagent 1. *o*-Aminodiphenyl (Timmel, Glaudemans & Currie, 1956); spray reagent 2. Hexosamine reagents (Partridge, 1948).

(ii) Amino acids (6N-HCl hydrolysates): solvent 1. Amyl alcohol–pyridine–water–diethylamine (Giri & Rao, 1953); solvent 2. Butanol–benzyl alcohol–water (Block & Bolling, 1951); spray reagent 1. 0·2% Ninhydrin (Meyer, 1957); spray reagent 2. Pauly diazo reagents for histidine (Sanger & Tuppy, 1951); spray reagent 3. Iodoplatinate reagent for cysteine and methionine (Toennies & Kolb, 1951).

(iii) Sialic acid: Solvent. Butanol–acetic acid–water; spray reagent. $AgNO_3$ in acetone followed by ethanolic KOH (Heimer, 1957).

Physical examinations

Absorption spectra. The ultraviolet absorptions of both materials were measured at 0·1% concentrations in saturated $CaCl_2$ soln.–ethanol (9:1, v/v) in a 1 cm. cell and a Unicam SP. 500 spectrophotometer. Infrared spectra were obtained with a Grubb–Parsons double-beam instrument having a rock-salt prism, by the KBr disk technique.

Electrophoresis. Some experiments were performed with the Tiselius apparatus (Hilger and Watts Ltd.); in acetate buffer, pH 5·8, $I = 0·1$. The mucin gives an intractable gel in this solvent and at best a semi-opaque gel at a concentration of about 0·5% was induced with difficulty to form suitable boundaries. Electrophoresis was carried out for from 3 to 5 hr. at 15 mA.

RESULTS

Physical properties. Both mucoids are, in water, clear gelatinous cohesive materials forming two phases. The oestrus preparation is physically indistinguishable from the initial secretion to the eye and the gel phase shows, like the native secretion, 100% elastic recoil [measured as described by Scott-Blair *et al.* (1941)]. The pregnancy mucoid is less firm and thick than the native mucous plug of pregnancy, but if dehydrated somewhat it becomes closely similar physically to the starting material.

A study of the sedimentation, viscosity and flow birefringence of these two purified specimens is reported in the next paper (Gibbons & Glover, 1959). Electrophoretic analysis was, as indicated, unsatisfactory, but with the oestrous mucoid the absence of material migrating more rapidly than the main component could be demonstrated. The absence of material migrating more slowly is not so certain, owing to the opacity of the specimen and the tendency of the mucin to adhere to the sides of the cell, giving a poorly defined descending boundary. The mucoid migrates anodically. The heterogeneity of crude oestrous mucin is readily detectable by electrophoresis, three or sometimes four components being visible.

The ultraviolet-absorption spectra showed no specific absorption maxima between 220 and 400 mμ. There was large absorption below 220 mμ and the pregnancy mucoid showed a small inflexion in the region of 270 mμ. The infrared-absorption spectra are shown in Fig. 2. They are closely similar, the main difference being in the slightly higher absorptions in the region of 1600 and 1400 cm.$^{-1}$ in the oestrous mucoid, probably attributable to carboxyl groups.

Chemical properties. Both mucoids in approximately 1 mg. amounts gave negative tests for tryptophan (glyoxylic acid test), ketoses (Seliwanoff test), heptoses (Dische, 1953) and uronic acids (Dische, 1947). After hydrolysis in 0·5 N-acid for 16 hr. neither sulphate nor phosphate (Allen, 1940) could be detected. The ferric hydroxamate test for *O*-acyl groups (McComb & McCreedy, 1957) was strongly positive for both mucoids. Both mucoids are completely precipitated at zero ionic strength by very low concentrations of cetyltrimethylammonium chloride and also by 0·01 M-flavianic acid. The mucoids are rapidly liquified at room temperature by dilute neutral sodium hypochlorite, as observed by Boyland (1946).

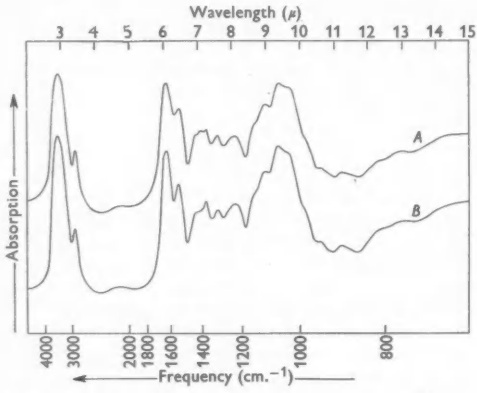

Fig. 2. Infrared-absorption spectra of *A*, oestrous mucoid, and *B*, pregnancy mucoid.

No qualitative difference between the oestrous and pregnancy mucoids was found. The sugars fucose, galactose, glucosamine and galactosamine were identified chromatographically. The following amino acids were likewise identified: serine, threonine, aspartic acid, glutamic acid, proline, glycine, alanine, valine, isoleucine, leucine, phenylalanine, lysine and arginine. Histidine, cysteine and methionine were shown to be absent by the use of specific spray reagents. Less than 0·2 % of lipid material could be extracted, though the crude mucins contain approximately 0·5–1·0 % of ether-extractable material. The identity of this lipid was not established, but it does not contain nitrogen, phosphorus, sugar or steroid in detectable amounts. It may be related to the material described by Lindahl & Nilsson (1957).

Chromatographic analysis of hydrolysates of the crude mucins reveals the presence of small amounts of glucose and mannose in addition to the sugars already mentioned. These two sugars are found in increased amount in the supernatants from the preparative ultracentrifuging procedure. The nature of the materials containing these two components is being investigated. The final supernatant after centrifuging at 103 000 *g* contains largely protein material. A sample of this material was precipitated with sodium sulphate at 20 % (w/v) concentration, and the resultant protein precipitate after dialysis was subjected to ultracentrifugal and electrophoretic examination, on the basis of which bovine serum albumin and globulin are tentatively identified.

Quantitatively two marked differences were found between the oestrus and pregnancy preparations. The amount of sialic acid in the pregnancy sample is significantly higher than in the oestrus material. A sufficient number of pregnancy (three) and oestrus (six) materials have been partially purified and examined to indicate that this difference is probably not fortuitous. The two mucoids contain about the same total amount of hexosamine but the glucosamine:galactosamine ratios differ. Hexosamine ratios have been obtained only for the two samples described here and it is uncertain whether this difference will be found generally between oestrus and pregnancy materials.

The complete analysis of the two mucoids is given in Table 2.

Both mucoids give a positive Morgan–Elson test for *N*-acetylhexosamine (Aminoff *et al.* 1952). The amount of colour developed increases with time of heating in 0·05 N-Na_2CO_3 to a rather indefinite maximum around 25–30 min., decreasing slowly thereafter (Fig. 3). The absorption spectrum of the

chromophore was found to be closely similar to that of authentic *N*-acetylglucosamine heated for 4 min. with 0·05 N-Na_2CO_3.

The rate at which free hexosamines and reducing sugars are produced by hydrolysis with 0·5 N-HCl at 100° is shown in Fig. 1.

Both mucins in their native state have weak but readily demonstrable lysozyme activity, equivalent to between 0·05 and 0·1 % of that of crystalline egg-white lysozyme (The Armour Laboratories, Eastbourne, Sussex). The purified preparations do not possess this enzymic activity, which can be found in appropriately increased concentration in fraction 1 (see Table 1). Egg-white lysozyme has no observable effect on the mucin (cf. Lawton *et al.* 1956).

Fractionation of crude oestrous mucin according to Heimer (1957) gave three fractions from the clot formed at pH 3·7: (i) material insoluble in acetate buffer (180 mg.; N, 8·2, galactose, 20·9, sialic acid, 11·1 %); (ii) material removed from solution in acetate buffer by the Sevag procedure (50 mg.; N, 12·6, galactose, 11·5, sialic acid, 6·3 %); (iii) material remaining in the final supernatant (6 mg.; N, 7·7, galactose, 14·9, sialic acid, 3·0 %). Two fractions were isolated from material fractionated by Curtain & Pye's (1955) procedure: (i) precipitated with ethanol, between 40 and 50 % (v/v) of ethanol (35 mg.; N, 6·4, galactose, 23·1, sialic acid, 12·5 %); (ii) precipitated at above 50 % (v/v) of ethanol (75 mg.; N, 6·5, galactose, 23·3, sialic acid, 12·8 %). Phenol-extraction of dried crude mucin yielded a phenol-insoluble material, part of which was water-soluble; analysis of this latter fraction gave N, 6·9, galactose 20·8, sialic acid, 13·5 %. Negligible amounts of sialic acid could be found in the fractions soluble in phenol. Enzymic digestion followed by phenol extraction gave a phenol-insoluble material (the whole of which was water-soluble), which contained: N, 5·8, galactose, 28·8, sialic acid, 12·6 %. Some sialic acid-containing material was in this instance soluble in phenol but almost the whole of it could be precipitated by addition of ethanol to the phenol supernatant to 12·5 % (v/v).

Hydrolysis of an oestrous mucoid preparation in 0·1 N-HCl at 20° for 48 hr. results in the material, initially a two-phase gel, slowly dispersing to give ultimately a clear viscous solution, rather like gastric mucin after peptic autolysis. The results of subsequent fractionation are shown in Table 3. It can be seen that the only component showing a marked decrease in amount is sialic acid, and in confirmation of this a chromatogram of the material not precipitated at 80 % (v/v) of ethanol concentration showed two spots only, both attributable, by comparison with an authentic sample, to sialic acid. No sugar or oligosaccharide was detected.

Table 2. *Analysis of oestrous and pregnancy mucoids*

Figures are given as g./100 g. of dry mucoid.

	Oestrous mucoid	Pregnancy mucoid
Galactose	27·5	28·1
Fucose	5·1	5·0
Glucosamine*	15·3	12·6
Galactosamine*	11·4	15·7
Sialic acid†	13·8	17·5
Acetyl‡	10·0	11·1
Ash	3·1	3·7
Nitrogen §	6·45	6·04
Serine	3·20	2·46
Threonine	6·28	5·45
Aspartic acid	1·85	0·73
Glutamic acid	2·63	1·77
Glycine	1·25	0·88
Alanine	2·02	1·70
Proline	3·98	2·40
Valine	1·59	1·77
Isoleucine	0·46	0·40
Leucine	1·19	0·86
Phenylalanine	1·12	1·14
Lysine	0·77	1·02
Arginine	1·38	1·38

* As base. † As the *N*-acetyl compound.
‡ As $CH_3 \cdot CO$. § Kjeldahl.

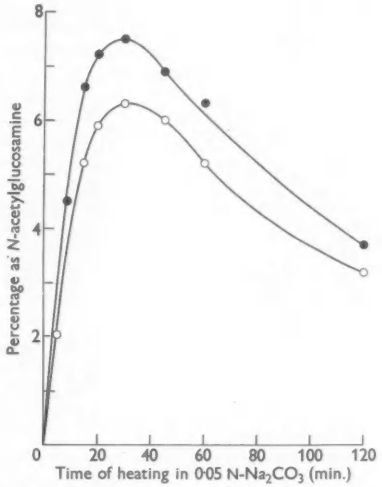

Fig. 3. *N*-Acetylhexosamine reaction. Oestrous mucoid (○) and pregnancy mucoid (●). The amount of colour formed is expressed in terms of an equal weight of *N*-acetylglucosamine heated in 0·05 N-Na_2CO_3 for its optimum time (4 min.).

Table 3. *Analysis of a sample of oestrous mucoid treated with 0·1 N-hydrochloric acid at room temperature for 48 hr. and subsequently fractionated with ethanol*

	N* (%)	Hexosamine† (%)	Galactose (%)	Reducing sugars‡ (%)	Fucose (%)	Sialic acid§ (%)
Initial material	6·62	20·0	21·7	39·8	5·1	13·4
Material after acid hydrolysis, fractionated with ethanol						
(*a*) 0–60% (v/v) ethanol precipitate	6·76	22·0	22·8	42·0	5·2	8·4
(*b*) 60–80% (v/v) ethanol precipitate	6·88	22·8	21·1	38·6	5·0	7·7

* Kjeldahl. † As base. ‡ As glucose. § As the *N*-acetyl comound.

DISCUSSION

It can be seen that the two preparations isolated are chemically mucoids of the 'blood-group substance' type or, according to Werner (1952), fucomucans. The analyses of Shettles, Dische & Osnos (1951) have already suggested the presence of this type of material in human mid-cycle cervical mucin. From the appearance of the mucoids isolated, there is every reason to believe that it is these materials which are responsible for the characteristic physical properties of epithelial mucin. The fractionation procedure has been designed to avoid physical or chemical damage to labile structures, for it has been found that such apparently harmless procedures as vigorous stirring or drying change the material physically. Shaking the mucin with amyl alcohol–chloroform mixtures, for example, results in the formation of a dense white gel in the organic layer from which mucin cannot be recovered in its native state; the aqueous layer is essentially devoid of mucoid. The purified mucoid may be reconstituted after careful freeze-drying over silica gel, to give apparently unchanged material, but the native secretion when freeze-dried becomes completely insoluble. It is suggested that the long thread-like mucoid molecules are extensively intertwined with one another in the gel phase and that any violent shearing results in their being torn apart and irreversibly broken by the applied physical force. This has been reported to occur to some other long-chain molecules (Tsvetkov & Frisman, 1945). In the native state, contaminating protein is probably enmeshed within the matrix of entangled mucoid threads, and part of the protein becomes denatured on drying. This would account for the behaviour of the freeze-dried native secretion.

The homogeneity of these two mucoid preparations cannot be regarded as established. It has been shown that they both sediment as one component and that the oestrous mucoid is probably electrophoretically homogeneous also; furthermore, known contaminants in the crude mucin (albumin- and globulin-like proteins, lysozyme, glucose- and mannose-containing material) are not detectable in the purified preparations. It is probable that extraneous material has been largely eliminated therefore, but it is possible that the preparations consist of a mixture of closely similar mucoids; indeed, since the starting material was collected from a number of different individual cows, this is probable. Despite the application of physical and immunological techniques, the establishment of homogeneity of mucoid preparations is not entirely satisfactory at present, and if the recent genetical theory due to Watkins & Morgan (1959) is correct it would seem that strict homogeneity in such preparations is likely to be unattainable. The use of individual animals with some immunological control would be a considerable improvement, however, and experiments along these lines are projected.

The amount of sialic acid in both these preparations is considerably higher than has been previously reported for this type of mucoid (Gibbons *et al.* 1955). It is unlikely, however, that this is due to contamination with sialoprotein since none of the procedures employed gave any indication of a separation of the galactose and sialic acid into different fractions. McCrea (1953) has shown that sialoprotein is readily soluble in phenol and the experiments described would almost certainly have revealed its presence. It is considered that the sialic acid is an integral part of the mucoid molecule, as isolated, and this view is supported by the rapid anodic electrophoretic migration of the oestrous mucoid. It is probably unwise to infer the presence of sialoprotein from the occurrence of large amounts of sialic acid in a mucous secretion.

The nature and distribution of the amino acids in these two mucoids do not suggest any extensive contamination with extraneous protein, for they are rather unlike that of proteins generally. The same amino acids are present as are found in the human blood-group mucoids (Aminoff, Morgan & Watkins, 1950; Annison & Morgan, 1952*a*, *b*; Gibbons & Morgan, 1954), with the single exception of phenylalanine, which these workers found to be absent and which is present in the two mucoids

examined here. The striking feature in the amino acid analysis (Table 2) is the large amount of the hydroxyamino acids, amounting together to about one-third of the total amino acids; the rather high proline content of these materials is also noteworthy. There is somewhat less of the acidic and somewhat more of the basic amino acids in the pregnancy sample than in the oestrus sample; otherwise the analyses of the two mucoids are quite similar. Threonine has been recognized as a major constituent of this type of mucoid since its isolation from a urinary mucoid in 1942 (Freudenberg, Walch & Molter, 1942); moreover Aminoff & Morgan (1951) find hydroxyamino acids to account for 34% of the amino acid nitrogen in their thoroughly purified blood-group A mucoid. Carsten & Kabat (1956) give amino acid analyses for samples of blood-group active mucoids isolated from hog gastric mucin and from human saliva, which are very similar to the analyses in Table 2; thus the distribution of amino acids reported here may be fairly typical of mammalian epithelial fucomucan. Two other published amino acid analyses of mucin, however, that of Folkes *et al.* (1950) (frog-spawn mucin) and Brand & Saidel (1946) (hog gastric mucin), do not show any marked resemblance to that of these two bovine cervical mucoids.

These two mucoids also resemble the human blood-group substances in that they contain the same sugar components, including sialic acid. Furthermore, the course of the *N*-acetylhexosamine reaction (Fig. 2) and the rate of hydrolysis of hexosamine in 0·5N-acid at 100° (Fig. 3) are similar to the behaviour of blood-group mucoids under the same conditions; free reducing groups, on the other hand, appear in 0·5N-acid hydrolysates somewhat more rapidly because of the relatively large amount of the very acid-labile component, sialic acid. This substance is rapidly destroyed in 0·5N-acid at 100°. The human substances also contain more fucose than the bovine mucoids, but Beiser & Kabat (1952) find that their blood-group active mucoids isolated from bovine gastric mucin are similarly low in fucose, so that fucose content is likely to be related to an inter-species difference in mucin structure. The lability of the fucose towards acids has not yet been investigated, but unlike the sialic acid it is not labile at pH 1 at room temperature. The greater part of the fucose in blood-group substances is labile at pH 3·0 at 100°.

The two bovine cervical mucoids are of considerably higher molecular weight (4×10^6) than any blood-group mucoid so far described (Gibbons & Glover, 1959).

It has been suggested (Gibbons, 1959) that the differing physical properties of epithelial mucin are related to the expansion factor of the constituent thread-like random-coil molecules. The two mucoids described here have been shown to have markedly different expansion factors (Gibbons & Glover, 1959), and it is probable that this in turn is related to the sialic acid component, since the sialic acid content constitutes the only major chemical distinction between the two mucoids so far as is known at present. Some confirmation of this view is given by the result of the 0·1N-acid hydrolysis experiment, since here the mucoid is converted from a two-phase gel into a viscous solution, which implies a marked increase in expansion factor, and there is concomitant release of some of the sialic acid residues. However, it is quite likely that some depolymerization may also be occurring, in which case the physical properties of the solution would change from this cause also. This point is being investigated. This experiment suggests that a major portion of the sialic acid is present as end group. It should be observed that Odin (1958) has concluded that the physical properties of the contents of human ovarian cysts are related, in the same way as cervical secretions, to their sialic acid contents.

The amount of acetyl group present in these mucoids is considerably greater than that required for acetylation of the amino groups of the hexosamine and the sialic acid, but is approximately correct if the sialic acid is mono-*O*-acetylated in addition. The positive ferric hydroxamate test supports this suggestion, but, as has been pointed out by Armstrong, Baddiley, Buchanan, Carss & Greenberg (1958), the test is not specific for *O*-acetyl groups; amino acid esters, which may well be present, also react.

The analyses in Table 2 account, within experimental error, for the whole of the material, and it is unlikely that a major constituent remains to be identified. The amino acids, sialic acid and hexosamines account for almost the whole of the nitrogen so that amide nitrogen is likely to be small. Quite large amounts of NH_4^+ ion are produced by acid hydrolysis but it is probable that most of this is due to the destruction of the sialic acid. The determination of hexosamine ratio gave a somewhat low overall recovery of hexosamine (88 and 90% respectively for the oestrous and the pregnancy mucoids), so that there is the possibility of an unidentified hexosamine being present. However, it is considered more probable that slight destruction of the hexosamines in 6N-hydrochloric acid accounts for the low yields.

Cervical mucin appears to be a mucous secretion containing mucoid relatively uncontaminated with extraneous material. The major foreign material appears to be protein, which may be eliminated relatively easily. Buccal and gastrointestinal mucin, in addition to contamination with food

particles, contains also acidic mucopolysaccharides (Werner, 1952; Glass, 1953; Pasternak, Kent & Davies, 1958); in salivary mucin a sialoprotein is present as a major constituent (McCrea, 1953; Curtain & Pye, 1955; Heimer & Meyer, 1956; Heimer, 1957). Urinary mucin contains a closely similar sialoprotein (Tamm & Horsfall, 1952). Cervical mucin does not contain appreciable amounts of uronic acid or ester sulphate; and experiments designed to isolate a sialoprotein constituent similar to that described by the above-quoted authors suggest that this material, if present, is a very minor constituent. It is possible that the small amount of mannose detected in the crude mucin originates from a material of this type (Gottschalk & Ada, 1956). Two other important sources of mucin, namely ovarian-cyst fluids and meconium, suffer from the disadvantage that they have been incubated at body temperature, often for long periods, a circumstance which may alter the physical and chemical properties of these secretions; in fact the ease with which soluble, relatively low-molecular-weight mucoid may be isolated from them rather suggests this. For these reasons, cervical mucin, apart from its intrinsic interest from the standpoint of fertility and of endocrinology, may well be a useful secretion upon which to study the biochemistry of mammalian mucin.

SUMMARY

1. A method for the isolation of undegraded mucoid from bovine cervical mucin is described.

2. Two samples of mucoid, one isolated from mucin obtained at oestrus and the other from pregnancy mucin, are shown to be largely free from known contaminants in the crude secretion, and to be the materials chiefly responsible for the physical properties of the native mucin.

3. The mucoids were found to consist of 75–80% of carbohydrate and 20–25% of amino acid residues, and to behave chemically in a manner similar to the human blood-group substances.

4. Fucose, galactose, glucosamine, galactosamine, sialic acid, threonine, serine, aspartic acid, glutamic acid, proline, glycine, alanine, valine, leucine, isoleucine, plenylalanine, lysine and arginine were identified chromatographically in both mucoids and estimated. The sialic acid content of the pregnancy mucoid (17·5%) is higher than that of the oestrous mucoid (14%).

5. The results of hydrolysis of oestrous mucoid in 0·1 N-hydrochloric acid at 20° suggest that part of the sialic acid is present as end group and that the sialic acid content is one of the factors affecting the physical properties of mucin.

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The Physicochemical Properties of Two Mucoids from Bovine Cervical Mucin

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A study of the phenomenon of flow-birefringence in solutions of macromolecules can yield information relevant to the size, shape and flexibility of the molecules in solution. Essentially this involves observation of the optical properties of a solution under shear, conveniently in a concentric-cylinder viscometer having a transparent base, fitted to a vertical optical bench. Consider a small segment of the annulus with the inner or outer cylinder rotating at constant speed: the flow lines will be along the annulus in a horizontal plane, the direction of the velocity gradient will lie radially across the annulus, also horizontally, and the line of sight will be vertical and at right angles to both. A schematic diagram of a suitable apparatus is given by Glover & Suffolk (1957).

Two factors are involved in the phenomenon of flow-birefringence: the optical and the hydrodynamic.

(1) *Optical factors.* A birefringent body possesses different refractive indices for light polarized in two directions at right angles. Linearly polarized light passed normally through such a body, in general, emerges elliptically polarized, and cannot be extinguished by rotation of an analyser. If the direction of polarization of incident light is coincident with, or at right angles to, the optic axis of the body, it emerges unchanged and can be extinguished by an analyser. Lines defining the direction of the optic axis are, when applied to a solution under shear, termed neutral lines. The factors which determine the position of the neutral lines in a flowing solution of birefringent molecules will be considered below. Their direction may be located by examining the flowing solution between crossed Nicol prisms. The smaller angle between the neutral lines and the flow lines is called the angle of isocline (χ). The amount of double refraction, that is the difference of refractive indices of the flowing solution for light with electric vectors parallel with and at right angles to the neutral lines, may be measured by means of a quarter-wave plate. It is referred to as the birefringence (Δn) and may be positive or negative.

Flow-birefringence of the type considered here is observed only in solutions of macromolecules which are geometrically anisotropic or which can be deformed in the applied velocity gradient. It will be seen that the effect of the velocity gradient is to cause an alignment, or apparent alignment, of the molecules with their longer axes at an angle between 45° and 0° to the flow lines; this alignment is the origin of the birefringence observed, which is the sum of that due to all the molecules in the field of observation.

The birefringence of a macromolecule arises from two causes: (i) the form or 'Wiener body' birefringence (Wiener, 1912), due only to the geometrical anisotropy of the molecule and to the difference in refractive indices between molecule and solvent, is a function of the square of the difference in refractive indices and is therefore always positive; the optic axis is coincident with the longer geometric axis. For a rigid ellipsoid or rod this is quite straightforward, but for the random-coil molecule the same considerations apply save that in this instance the boundary between the solute particle and solvent is gradual and not abrupt; thus some sort of average difference in refractive indices must be taken (Tsvetkov, 1957). Furthermore, it is necessary that the molecule be deformed into a geometrically asymmetric shape by Brownian motion or by the applied velocity gradient; thus form-birefringence for molecules of this type will be a function of velocity gradient. (ii) The intrinsic birefringence is due to variation in polarizability of the constituent chemical bonds in different directions. There is no presumptive reason why the optic axis of the intrinsic birefringence should coincide with the geometric axis of a rigid body, but it will nevertheless behave as though it does, due to the symmetry of distribution of orientations of the molecules about an axis parallel to the flow lines. In the random-coil molecule, there will be intrinsic birefringence in the deformed condition due to the favoured alignment of parts of the chain in the direction of elongation. This is analogous to strain-birefringence in an isotropic body; the optic axis will be coincident with the geometric axis of distortion. The intrinsic birefringence of a random-coil molecule as well as its form-birefringence will therefore be a function of velocity gradient. The position is not quite so straightforward as this, however, and the question of the position of the optic axis of the intrinsic birefringence of a molecule of this type is considered in detail in the Discussion.

(2) *Hydrodynamic factors.* Rigid molecules in solution when subjected to shear in the type of apparatus indicated will rotate about an axis in the line of sight. If geometrically anisotropic they will spend a longer portion of their time of revolution in a position in which their longer geometric axes are along the lines of shear than they do in a position with their axes across them (Trevelyan & Mason, 1951). This introduces some degree of order into the sheared solution, in opposition to the effect of Brownian motion which tends to give a random distribution of orientations. As a result there is an apparent (statistical) alignment of the molecules in the solution with their geometric axes at some angle to the flow lines. At low velocity gradients, where the effect of Brownian motion is relatively large, this angle will approach 45°; as the velocity gradient increases it will approach 0° asymptotically. The behaviour of random-coil molecules, which are not rigid, is more complicated, and it is necessary to consider two limiting conditions, one in which the molecule passes readily from one configuration to another (low internal viscosity), and another in which the energy barriers between one configuration and another are large (high internal viscosity). A molecule of low internal viscosity, as pointed out by Cerf (1951*a*), will behave like an elastic sphere, or liquid drop, and will be distorted at low gradients into an ellipsoid having its major axis at 45° to the flow lines and will become successively more elongated and more closely aligned with the flow lines as the gradient increases (Taylor, 1934; Bartok & Mason, 1958). A molecule with high internal viscosity, on the other hand, having assumed an ellipsoidal configuration, will behave in much the same way as a rigid body. Real molecules probably show behaviour intermediate between these two cases. More detailed and sophisticated treatments of the case of the random-coil molecule are given by Kuhn & Kuhn (1946) and Cerf (1951*a*, *b*; 1958).

The variation of both χ and Δn with the velocity gradient, G, will in general be of the same form for both the rigid molecule and for a molecule of the deformable, random-coil type; typical curves resemble those in Figs. 3 and 4. In general, rigid molecules give larger birefringences, which increase more rapidly with increasing velocity gradient than do random-coil molecules, but this criterion is inadequate for unequivocal differentiation. Edsall (1942), Lawrence, Needham & Shen (1944) and Cerf & Sheraga (1952) have published reviews on the subject to which the reader is referred for a more complete treatment.

The preceding paper (Gibbons, 1959*a*) describes the isolation of two mucoids from bovine cervical mucin. The flow-birefringence of solutions of these two mucoids has been examined and some anomalous results have been obtained. An explanation of these anomalies is offered and it is inferred that these materials are flexible thread-like molecules of the random-coil configuration. Advantage is taken

of this result to determine the molecular weights and relative expansion factors of these two samples from sedimentation coefficients and intrinsic viscosities. The results give some indication of the factors underlying the difference in physical properties of the native secretions. For a description of this difference see Scott-Blair, Folley, Malpress & Coppen (1941).

EXPERIMENTAL

Materials

Mucoids. These were obtained from two bulked samples of bovine cervical mucus, one obtained from cows in oestrus and the other from pregnant animals. Ultracentrifugally homogeneous mucoid was prepared from each sample as described by Gibbons (1959*a*).

Solvents. Two solvents were used: (1) aqueous saturated calcium chloride solution–ethanol (9:1, v/v); (2) lithium citrate–citric acid buffer, pH 5·8 at 20°, containing 0·2 M-lithium chloride, 8 mM-trilithium citrate, 2 mM-citric acid and 10% (v/v) of ethanol. Solvent (2) was used for ultracentrifugal and viscometric studies and its viscosity was determined in the temperature range 20–30° with calibrated Ostwald viscometers; the density in this temperature range was also determined, by Kohlrausch's method (Short, 1955). Solvent (2) was employed owing to the difficulty of obtaining single-phase solutions in the usual phosphate or acetate buffers.

Methods

Flow-birefringence. The apparatus used was based on that of Ogston & Stanier (1953) and is described by Glover & Suffolk (1957). Observations were made at room temperature and in green light (λ 5461Å) isolated from a mercury arc. The apparatus is designed so that the rate of shear is equivalent to the rate of rotation of the outer cylinder in rev./min., which was measured electronically. It has been observed that mucin may be degraded by subjection to high shear rates, consequently only relatively low rates (<500 sec.$^{-1}$), particularly with concentrated solutions, were employed.

Viscosity. The flow-birefringence apparatus may be used as a Couette viscometer. It was modified by installation of a low-speed electric motor in order to enable rates of shear down to 0·2 sec.$^{-1}$ to be applied.

Sedimentation coefficients. A Svedberg oil-turbine ultracentrifuge having the bar-schlieren optical system of Baldwin (1953) was employed. Plates were measured with a travelling microscope and sedimentation coefficients obtained and corrected to water at 20° as described by Svedberg & Pedersen (1940). Concentrations of mucoid from 0·5 to 0·075% were examined.

Partial specific volumes. These were determined pyknometrically in a 10 ml. density bottle, with solutions of mucoid in buffer at concentrations in the region of 0·25%. The measurements were made in a constant-temperature room at 20°, about 4 hr. being allowed for temperature equilibration. Somewhat lower concentrations than is usual were weighed as it is extremely difficult to avoid inclusions of air if higher concentrations are employed. The accuracy of the figures quoted (Table 1) is probably about ±1%.

Results

Flow-birefringence. The variation in angle of isocline and birefringence with velocity gradient is most conveniently shown graphically. Figs. 1–4 show the results for both the oestrus sample and pregnancy sample in solvent (1). Fig. 5 shows both birefringence and angle of isocline curves for the oestrus sample in solvent (2). The curves in Figs. 1 and 2 are anomalous, whereas those in Figs. 3 and 4 are at first sight normal, but there is, at the higher concentrations, a very rapid change in angle of isocline with velocity gradient at the lowest rates of shear examined. The curves in Fig. 5 are again anomalous.

Viscosity. The specific viscosities, $([\eta/\eta_0]-1)/c$, were plotted against concentration and extrapolated to infinite dilution (Fig. 6); values obtained for specific viscosities at infinite dilution are given in Table 1. At the concentrations (0·12–0·008%) and rates of shear (0·2–14 sec.$^{-1}$) investigated, the mucoid solutions behaved as Newtonian fluids.

Sedimentation coefficients. The reciprocals of the sedimentation coefficients ($1/S_{20,w}$) were plotted against concentration (Fig. 7) and extrapolated to infinite dilution. As may be seen, there is indication of slight curvature in the graph for both mucoids but the best straight line has been preferred for extrapolation purposes. If the curvature is real, the figures quoted for $S^0_{20,w}$ (Table 1) may be slightly low. The schlieren diagrams are shown in Fig. 8.

DISCUSSION

Flow-birefringence

An inversion of sign of the birefringence with increasing velocity gradient (Fig. 2) has already been observed, by Tsvetkov (1955), in solutions of high-molecular-weight polystyrene in dioxan. It has been shown that the form-birefringence of a random-coil macromolecule, such as polystyrene, is a different and more slowly varying function of the velocity gradient than the intrinsic birefringence (Frisman & Tsvetkov, 1958; Copic, 1956, 1957), and it can be seen that if the form-

Table 1. *Physical constants of the two bovine cervical mucoids*

	$10^{13}S^0_{20,w}$ (sec.)	$[\eta]$ (dl./g.)	$\bar{v}$ (ml./g.)	$10^{-6} \times M$
Oestrus sample	20	7·4	0·63	4·16
Pregnancy sample	25	3·2	0·64	3·93

birefringence is, at low velocity gradients, of greater magnitude than the intrinsic birefringence and opposite in sign, the birefringence will, at small gradients, be positive but will become negative as the velocity gradient is increased. Tsvetkov (1957) interprets the curves in this way and adduces some further supporting evidence for this view. No

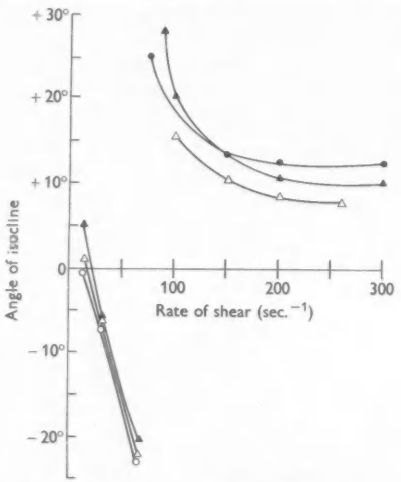

Fig. 1. Angle of isocline of pregnancy mucoid in aqueous saturated calcium chloride solution–ethanol (9:1, v/v) as a function of rate of shear. Concentrations: 1·2% (○); 0·6% (△); 0·3% (▲); 0·15% (●).

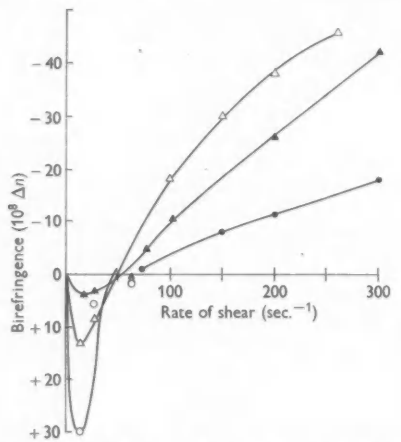

Fig. 2. Birefringence of pregnancy mucoid in aqueous saturated calcium chloride solution–ethanol (9:1, v/v) as a function of rate of shear. Concentrations are as given in Fig. 1.

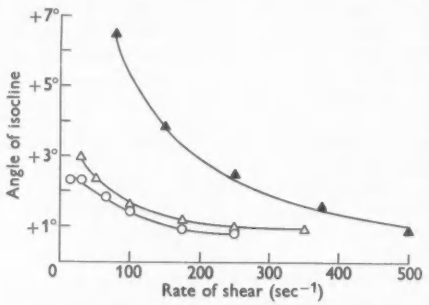

Fig. 3. Angle of isocline of oestrous mucoid in aqueous saturated calcium chloride solution–ethanol (9:1, v/v) as a function of rate of shear. Concentrations: 0·5% (○); 0·4% (△); 0·1% (▲).

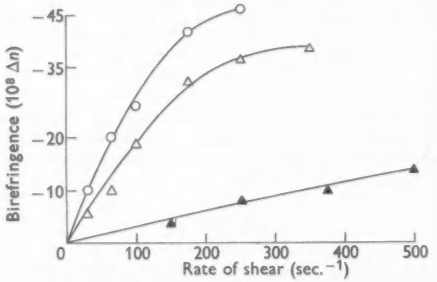

Fig. 4. Birefringence of oestrous mucoid in aqueous saturated calcium chloride solution–ethanol (9:1, v/v) as a function of rate of shear. Concentrations are as given in Fig. 3.

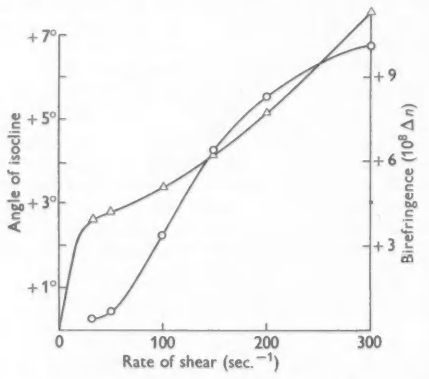

Fig. 5. Oestrous mucoid in ethanolic lithium citrate–citric acid buffer, pH 5·8, concentration 0·3%. Birefringence (△, right-hand ordinate) and angle of isocline (○, left-hand ordinate) as functions of rate of shear.

explanation has been offered for the corresponding angle of isocline curves (Fig. 1), which are again similar in form to those given by Frisman (1958) for high-molecular-weight polystyrene in dioxan. They may be interpreted if the assumption is made that the optic axis of the form-birefringence is coincident with the geometric axis of the deformed molecule, whereas that of the intrinsic birefringence is at a small angle to it. The variation of χ with increasing distortion of the molecule may then be investigated by imagining the two sources of birefringence to be separate and then using the relation of Sadron (1938) to sum them. Thus

$$\tan 2\chi = \frac{F(\beta)\,\theta_f \sin 2\phi_f - F'(\beta)\,\theta_i \sin 2\phi_i}{F(\beta)\,\theta_f \cos 2\phi_f - F'(\beta)\,\theta_i \cos 2\phi_i},$$

θ_f and θ_i are the form and intrinsic birefringences, and ϕ_f and ϕ_i are the orientations of the optic axes of the form and intrinsic anisotropies to the flow lines. β is a function of G, and for a given polymer is a constant multiple of it. F is a function for which different values are given by Copic (1957) and Frisman & Tsvetkov (1958), but in either case it varies more slowly with increasing β than F', which is $\beta(1+\beta)^{\frac{1}{2}}$. Consider the case where $\phi_f < \phi_i$; then $\sin\phi_f < \sin\phi_i$, but $\cos\phi_f > \cos\phi_i$.

When β is low, F and F' have similar values so that as θ_f is numerically greater than θ_i both numerator and denominator will be positive and $\tan 2\chi$ will be positive. As β is increased, $F'(\beta)$ increases more rapidly than $F(\beta)$, so that at some point the numerator becomes zero; but because of the inequalities set out above, the denominator is still positive. At this point $\tan 2\chi = 0$, $\chi = 0$. Further increase of β results in $\tan 2\chi$ becoming negative, since the numerator will be negative whereas the denominator is still positive. The point at which the denominator is zero is then approached; here the numerator is negative and finite and $\tan 2\chi = \pm\infty$, so that χ now descends to $-45°$. Further increase in β gives both numerator and denominator negative, hence χ is positive and decreases from 45° asymptotically to zero.

Under the conditions given then, the curves of χ against G would have the form found experimentally by Frisman (1958) and in this investigation. The effect on the angle of isocline as ϕ_i approaches ϕ_f, that is, becomes closer to the geometric axis of

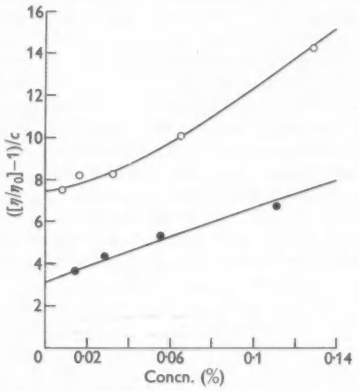

Fig. 6. Plot of intrinsic viscosity against concentration at rates of shear between 0·2 and 14·0 sec.$^{-1}$. ○, Oestrous mucoid; ●, pregnancy mucoid.

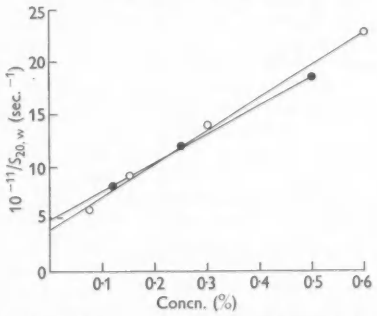

Fig. 7. Plot of $1/S_{20,w}$ against concentration. ○, Oestrous mucoid; ●, pregnancy mucoid.

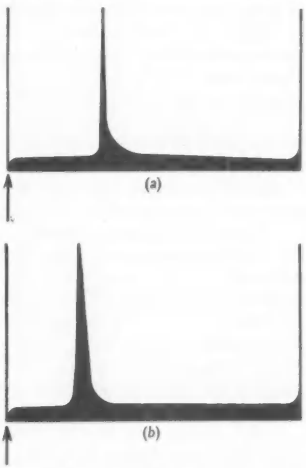

Fig. 8. Tracings of the ultracentrifuge schlieren diagrams for (*a*) oestrous mucoid, 0·3%, and (*b*) pregnancy mucoid, 0·5%; in ethanolic lithium citrate–citric acid buffer, pH 5·8, at 270 000 *g* and 25°. Exposures 15 min. after reaching full speed. The menisci are indicated by arrows.

the deformed molecule, is to reduce the range of values of velocity gradient over which $\tan 2\chi$ descends from a positive value through zero to $-\infty$ and from $+\infty$ to the same positive value; that is $d\chi/dG$ becomes very large in this region. When ϕ_i coincides with ϕ_f this part of the curve theoretically becomes vertical, but at this point the birefringence will now be zero, so that this part of the curve will be unobservable. Quite a small inclination of ϕ_i to ϕ_f will explain the observed peculiarities so that their origin may be sought in a disturbance which is quantitatively small.

Experimentally it is found that the birefringence is zero at velocity gradients in the region where χ is $\pm 45°$. From the equation given by Sadron (1938) it can be seen that this implies that the sine terms are small compared with the cosine terms. At this point therefore both ϕ_f and ϕ_i are small and the axis of the deformed molecule is close to the flow lines.

A possible source of the discordance between the optic axes of the intrinsic anisotropy and the geometric axis lies in the peripheral portions of the molecule. In both translation and shear the greater part of a random-coil type of molecule may be regarded as immobilizing the solvent within it. The semi-empirical relations of Kuhn & Kuhn (1952) indicate that the radius of the equivalent sphere in shear depends primarily upon the number of statistical chain elements in the molecule and also (but to a much smaller degree) on the ratio of the length to thickness of the chain element. In most cases of interest (that is, where the number of statistical chain elements is large), the equivalent sphere will comprise over 95 % of the molecule. The velocity gradient within the greater part of the molecule will therefore be sensibly zero, whereas in the peripheral parts it will increase radially. The chain elements in the peripheral regions will therefore tend to be orientated in this velocity gradient. The orientation of statistical chain elements due to a velocity gradient within a random-coil molecule has not received much consideration in the past since the effect is likely to be quantitatively small in most cases of interest. Cerf (1954) has considered the problem with the simplifying assumption that the material within the domain of the molecule behaves as a homogeneous liquid, the dynamic birefringence being assumed to be at 45° to the internal flow lines. Rigorous treatment of the problem of the differential orientation of chain segments involves complicated hydrodynamic and statistical considerations, but the following reasoning will give a qualitative indication of the result of this effect. The molecule may be approximated to an impermeable elastic sphere, in the immediate vicinity of which there are a number of very much smaller free-draining chain molecules. The sense in which these small molecules will be orientated is shown diagrammatically in Fig. 9, both for the sphere (to which the macromolecule may be approximated at vanishingly small gradients) and for an ellipsoid of revolution (into which the macromolecule will be deformed at higher gradients). In both cases the macromolecule will tend to rotate as shown, giving rise to local velocity gradients in the periphery, which will cause orientation of the small molecules as indicated. With the sphere, considerations of symmetry indicate that the local velocity gradient at the periphery of the sphere, due to its rotation, will be proportional to $\cos 2\theta$ (Fig. 9), and therefore the resultant orientation of the small molecules will be at 45° to the flow lines. With the ellipsoid of revolution, which is here shown with its major axis aligned with the flow lines, the resultant orientation of the small molecules is not obvious by inspection but it can be seen that it will in general be at non-zero angle to the major axis of the ellipsoid, and the result is the same if the major axis of the ellipsoid is not aligned with the flow lines.

The important conclusion is that the parts of the chain in the peripheral regions will be orientated in a different manner from those parts in the main bulk of the macromolecule, where there will be a preferred direction of orientation along the major axis of the ellipsoid. A component of the birefringence will be thus introduced which is not coincident with this axis. If the parts of the molecular chain in the peripheral regions are

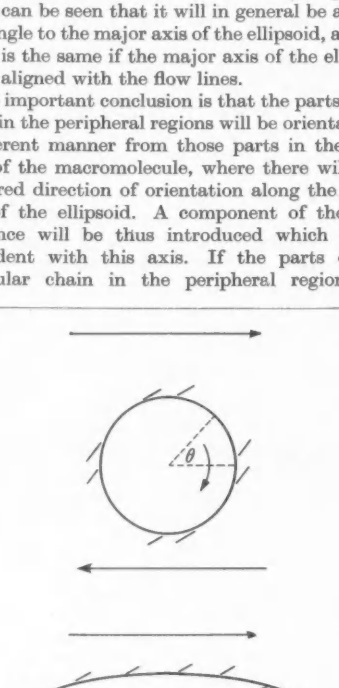

Fig. 9. Diagram to show the orientation of the peripheral portions of a random-coil molecule in a flow gradient.

assumed to behave as separate free-draining coil molecules, the amount of birefringence from this source will increase as the square of the local velocity gradient (Kuhn & Kuhn, 1945), and may reasonably be considered as part of the intrinsic anisotropy of the molecule. The result of the contribution of the peripheral parts of the molecule (which will be quantitatively small compared with the overall intrinsic birefringence), will be that the optic axis of intrinsic birefringence will be rotated by a small angle to the geometric axis in a direction away from the flow lines. This is a result of the absence of any sharply defined boundary between the domain of a random-coil molecule in solution and the solvent.

Thus far a single molecule has been considered, implying infinitely dilute solutions. It can be seen that with increasing concentration the point on the G axis where $\chi = 0$ moves towards lower values of G. Frisman & Tsvetkov (1958), who also observed this, suggest that as concentration increases the average difference in refractive index between a molecule and the solvent decreases owing to the increasing amount of overlap between the domains occupied by adjacent molecules. As a result, the contribution due to the form-birefringence does not increase linearly with concentration once the concentration has reached the point where there is contact between adjacent molecules. The intrinsic birefringence, on the other hand, is unaffected by this factor. Other points should be considered, however; for example, increasing concentration will result in higher local velocity gradients, due to molecular rotations, for a given macroscopic value of G, which will result in correspondingly greater molecular distortion; also, at higher concentrations, the permeability of the molecules must increase.

The birefringence curves for the oestrous mucoid in solvent (1) do not show the anomalies of the pregnancy sample, nevertheless the angle of isocline does, in the more concentrated solutions, increase very sharply at low gradients (Fig. 3), showing some resemblance to the curves for pregnancy mucoid at somewhat higher gradients (Fig. 1). It is possible therefore that the inversion of sign of birefringence and angle of isocline may be occurring at rates of shear lower than those measured, and where the birefringences are too small for detection. This is a possibility which should be considered when extrapolating the angle of isocline to zero rates of shear (Cerf & Sheraga, 1952). The birefringence is in this case negative, so that it is predominantly due to intrinsic birefringence; it may therefore be concluded that the form-birefringence is smaller than with the pregnancy mucoid. This could be due to a difference in molecular weight but the sedimentation and viscosity data (see below) do not suggest this. It appears from equation (3) in Tsvetkov (1957) that the form-birefringence will vary inversely as the 'volume' of the domain of the macromolecule, that is, inversely as the cube of the molecular-expansion factor (Flory, 1953). A similar relation is implicit in Copic's (1957) calculations. It will be seen that the sedimentation and viscosity data confirm that the oestrous mucoid is a more expanded molecule than the pregnancy mucoid. Gibbons (1959*b*) has suggested that this difference is chiefly responsible for the differences in physical properties of the native secretions.

The curves in Fig. 5 for the oestrous mucoid in solvent (2) are again anomalous. Similar curves were found by Gray & Alexander (1949) for aluminium laurate in benzene, but these authors found measurable birefringences at zero shear rates, which were not unequivocally observed in this case. Solvent (2) is a much poorer solvent than solvent (1) so that form-birefringence may be appreciable in this case; it should be noted that the birefringences are now positive. If the same sort of reasoning as has been used to elucidate the form of the birefringence curves in Figs. 1 and 2 is applied to where the form and intrinsic birefringences are of the same sign, curves similar to those in Fig. 5 can be obtained. A change of sign of the intrinsic birefringence implies extensive and intimate association of solvent with solute in one of the solvents; in view of the composition of solvent (1) and the known affinity of Ca^{2+} ions for sugars this is not unlikely. Kwart & Shashoua (1958) give other evidence for the interaction between mucin and alkaline-earth ions. The curves may also be accounted for if large aggregates are present. Although there is no indication of such aggregates from ultracentrifugal analysis, Joly (1958) has found evidence that they may arise as a result of the applied shearing stresses. The interpretation of these curves may be left open for the present.

It should be noted that the evidence presented can be most reasonably interpreted in terms of the flexible-coil molecule as a model. Polystyrene, which behaves similarly, is known to be of this form. This suggests that the mucoids examined here are also of this form, though a moderate degree of chain-branching would probably not affect their properties appreciably. This is an important conclusion since it becomes possible to make use of the rather extensive knowledge now available of the physicochemical and thermodynamic properties of this type of molecule (Flory, 1953).

Molecular weights

We have determined the molecular weights by means of the relations of Kuhn & Kuhn (1950), as

modified by Kuhn, Moning & Kuhn (1953), assuming that the number of statistical chain elements is large. This is justified in view of the large molecular weights and relatively low intrinsic viscosities.

With symbols defined as follows (Kuhn *et al.* 1953): λ_t, translational resistance factor; N_m, number of statistical chain elements in the molecule; A_m, length of a statistical chain element; L, extended length of molecule; N_A, Avogadro's number; S_0, sedimentation coefficient at infinite dilution; η_0, viscosity of solvent; ρ, density of solvent; $\bar{v}$, partial specific volume; $[\eta]$, intrinsic viscosity; ϕ, volume fraction of solute; r, effective radius of molecule:

$$S_0 = \frac{M(1-\bar{v}\rho)}{\eta_0 L N_A \lambda_t}.$$

Where N_m is large, $1/\lambda_t = 0{\cdot}136\sqrt{N_m}$. Putting $L = A_m N_m$ this becomes

$$S_0 = \frac{M(1-\bar{v}\rho)\ 0{\cdot}136\sqrt{N_m}}{N_A \eta_0 A_m N_m} = \frac{M(1-\bar{v}\rho)\ 0{\cdot}136}{N_A \eta_0 A_m \sqrt{N_m}}.$$

Considering now the intrinsic viscosities,

$$[\eta] = 2{\cdot}5\ \phi \text{ (Einstein, 1911)} = 0{\cdot}025\ \tfrac{4}{3}\pi r^3 \frac{N_A}{M}.$$

Taking r to be $0{\cdot}46\ A_m\sqrt{N_m}$ (Kuhn, 1948), we have

$$A_m\sqrt{N_m} = \frac{1}{0{\cdot}46}\left(\frac{30[\eta]\ M}{\pi N_A}\right)^{\frac{1}{3}},$$

where $[\eta]$ is expressed in decilitres/g. Therefore

$$S_0 = \frac{M^{\frac{2}{3}}(1-\bar{v}\rho)\ 0{\cdot}0625}{N_A^{\frac{1}{3}}\ \eta_0\left(\frac{30[\eta]}{\pi}\right)^{\frac{1}{3}}}.$$

This relation is numerically almost identical with the Flory (1953) relationship, if the constant $\phi^{\frac{1}{3}}P^{-1}$ is taken to be $2{\cdot}5 \times 10^6$. If Stokes' law is assumed in translation, and molecular weights are calculated from equation 14 in Ogston (1953) putting $J = 1$, somewhat higher values for M are obtained. The relevant data are set out in Table 1. It is important that the molecular weights reported refer most probably to the lithium salt (Kent, Record & Wallis, 1957), assuming the acidic groups in the mucoids to be sialic acid residues and that the pK_A of combined sialic acid is of the same order as that of the free acid (Svennerholm, 1957).

Expansion factor

It can be seen that the molecular weights of the two samples are not significantly different, but their intrinsic viscosities differ by a factor of about 2. This implies that under the conditions of experiment the oestrous mucoid is about 25% more expanded than the pregnancy mucoid (Flory, 1953). This figure may not have much quantitative significance when applied to the mucoids as secreted, but the oestrus secretion is a visco-elastic gel containing about 0·5% of non-diffusible solid, whereas the pregnancy mucin is a thick almost rubbery plasto-elastic material, the concentration of non-diffusible solids being a good deal higher (3–4%). A higher expansion factor for oestrus material is therefore indicated (Gibbons, 1959*b*), although both materials in the native state are, in aqueous solvents, below their Θ point (Flory, 1953) and form two phases when mixed with water. The pregnancy secretion usually swells somewhat in water, suggesting that the presence of salts in the secretion affects the expansion factor. The presence of extraneous material, such as proteins, may also affect it, for, though there is no very obvious change on adding bovine serum albumin or globulin to mucin, protamine produces a rather dense gel with both mucoids.

The factors affecting the physical properties of mucin are considered in more detail by Gibbons (1959*b*); and the chemical properties of these two mucoid specimens are described in the preceding paper (Gibbons, 1959*a*).

SUMMARY

1. Sedimentation, viscosity and flow-birefringence measurements have been carried out on two samples of bovine cervical mucoid.

2. Anomalous flow-birefringence curves have been found which have been interpreted as indicating molecules of the random-coil form.

3. Sedimentation and viscosity measurements have been used to estimate molecular weights, assuming a random coil having a large number of statistical chain elements. Molecular weights of the order of 4×10^6 have been found for both materials.

4. The specific viscosity of the sample isolated from animals in oestrus is about twice that of the sample isolated from pregnant cows, indicating that the oestrous mucoid is more expanded than the pregnancy mucoid.

5. The bearing of these data on the physical properties of the native secretion is briefly discussed.

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Fractionation of a Leucocidin from *Staphylococcus aureus*

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Staphylococcus produces at least three leucocidins, of which that originally described by Panton & Valentine (1932) is of particular interest since it attacks only white blood cells and not, like the other staphylococcal leucocidins, red blood cells as well. It is also specific to humans and rabbits. Some of the biological properties of this substance have been described by Gladstone & van Heyningen (1957). In the present paper the production of culture filtrates with high leucocidin activity is described and it is shown that this activity depends upon at least two proteins. These have been highly purified.

MATERIALS AND METHODS

Culture filtrates

Organism. The V8 strain of *Staphylococcus aureus* (Gladstone & van Heyningen, 1957) was used. A stock preparation on a tryptic meat–agar slope was made every month from freeze-dried organisms and cultured overnight on further tryptic meat–agar slopes to provide inocula for the production of culture filtrates.

Comparison of media for production of leucocidin. This was done in rocking T-tubes (van Heyningen & Gladstone, 1953) at 37° with 20 hr. growth. The cultures were clarified by centrifuging and assayed for leucocidin. Growth was estimated by suspending the organisms in 0·85% NaCl soln. and measuring their turbidity.

Large-scale production of leucocidin. This was done by Mr R. Elsworth and Mr R. Telling at the Microbiological Research Establishment, Porton, Wilts. The medium was that described by Gladstone & van Heyningen (1957), with the substitution of Oxoid Casamino acids and a diffusate of Oxoid yeast extract for the corresponding Difco products. Medium (80 l.) was inoculated with 20 l. of seed (prepared in turn from a 1 l. shake flask inoculated from a tryptic meat–agar slope) and the culture grown in a stainless-steel vessel with bottom aeration and stirring. When the maximum leucocidin titre was reached (8–10 hr.) the culture was cooled to below 10°, clarified by centrifuging and the supernatant sterilized by filtration. Solid $(NH_4)_2SO_4$ was then stirred in to give a 3·5 M-solution and the precipitated material collected by filtration through Whatman no. 1 paper and Hyflo-Supercel. The Hyflo-Supercel and

* Member of the Scientific Staff Medical Research Council.

precipitate were scraped off the paper and extracted with 0·1 M-sodium phosphate buffer, pH 6·7. Solid $(NH_4)_2SO_4$ was added to saturate the extract and the precipitate collected by centrifuging and stored as a slurry in saturated $(NH_4)_2SO_4$ soln. This material will be known as the culture-filtrate slurry.

Preparation of yeast diffusate. Oxoid yeast extract (120 g.) was suspended in 85 ml. of water and dialysed in cellophan tubing (1·5 cm. × 75 cm.) against 770 ml. of distilled water for 24 hr. with mechanical agitation of both sac and diffusate. The sac developed considerable tension but rarely burst. The final diffusate contained 7·5 g. of solids/100 ml.

Sterilization of the media. In both the large- and the small-scale experiments medium was sterilized by autoclaving at 15 lb./in.^{2} for 20 min. When glucose was used it was added as a sterile solution after autoclaving.

Assay of leucocidin

A simple assay procedure has been developed, based on the observation of Jensen & Maaløe (1950) that leucocidin abolishes the ability of leucocytes to reduce the blue dye, phenol-indo-2:6-dichlorophenol, to a colourless compound in the presence of KCN (cf. reduction of methylene blue, Neisser & Wechsberg, 1900). Macrophages are preferred to leucocytes as they can be kept alive *in vitro* for longer periods.

Serially diluted leucocidin solutions were incubated with the macrophages, the dye and KCN were added and incubation was continued until a clear distinction could be made between those solutions which were blue (in which the macrophages were dead) and those which were white (where the dye had been reduced by the living cells). The method can be used to determine directly the leucocidin activity of a solution or its antibody-combining power.

Minimum leucocidal dose (MLeD). This unit is defined as the smallest amount of leucocidin in 1 ml. which will kill macrophages under these assay conditions.

Test dose (L^+). This unit is defined as the smallest amount of leucocidin in 1 ml. in the presence of 1 unit of antitoxin which will kill macrophages under these assay conditions. Solutions of leucocidin differing in concentration by 25% can be distinguished by the MLeD test, and the L^+ test distinguishes between leucocidin concentrations differing by 10%.

Reagents. (*a*) Gelatin–phosphate buffer, pH 7·2, contained (in 1000 ml.) 9·5 g. of Na_2HPO_4, 2·72 g. of NaCl, 5 g. of gelatin, 18·5 ml. of N-HCl and 3·6 g. of glucose. The gelatin (Harrington Bros. Ltd., London) was purified by dialysing a concentrated gel against distilled water. The dry wt. was then determined and the gels were used in the preparation of the buffer. To dissolve the gels it was necessary to boil the solution and the glucose was added after the hot solution had cooled. (*b*) Phenol-indo-2:6-dichlorophenol (referred to as the dye; British Drug Houses Ltd., Poole, Dorset) was dissolved in gelatin–phosphate buffer to give a solution with $E^{1\,cm.}_{600}$ 6·0. (*c*) KCN (110 mg./100 ml. of gelatin–phosphate buffer). (*d*) Staphylococcal antitoxin was a pepsin-refined antiserum kindly provided by Miss Mollie Barr of the Wellcome Research Laboratories. It was standardized by the method of Gladstone & van Heyningen (1957). The assays reported in this paper have been made with a single batch of antitoxin CPP 76/63.

Macrophage suspension. Macrophages were taken from the peritoneal cavity of rabbits 4–6 days after the injection of liquid paraffin. The suspension of macrophages in the gelatin–phosphate buffer was filtered through muslin and standardized by selection of such a concentration that 2 vol. would reduce the dye to a colourless compound under the conditions described below, whereas 1 vol. would not.

Method. Logarithmically falling dilutions of leucocidin were prepared in the gelatin–phosphate buffer. In the determination of the MLeD these were assayed directly; in the determination of the test dose a constant amount of antitoxin was then added and the mixtures were assayed. In most of the assays recorded in this paper the mixtures contained 0·5 unit of antitoxin/ml.

In the following stages it was found convenient to use calibrated Pasteur pipettes. A volume (2 drops) of the leucocidin dilution was withdrawn and incubated with 2 drops of macrophage suspension at 37° for 15 min. Dye (1 drop) and 1 drop of KCN were then added and the incubation was continued for 5–10 min. The end point was the tube containing the greatest dilution of leucocidin which retains the blue colour.

Chromatography

Hydroxylapatite. This was prepared and set up in columns by the method of Tiselius, Hjertén & Levin (1956), except that the two periods of heating in mM-phosphate buffer, pH 6·7, were omitted and the material was stored in 0·1 M-sodium phosphate buffer, made by diluting the 0·5 M-phosphate buffer, pH 6·7, described below.

Fractionation of crude leucocidin on hydroxylapatite was by stepwise addition of phosphate buffers of increasing molarity. A stock 0·5 M-phosphate buffer was made by addition of NaOH to 1 mole of NaH_2PO_4, to give pH 6·7, and dilution to 2 l. Buffers of lower concentration were made by dilution without pH adjustment.

Carboxymethylcellulose. This was prepared and set up in columns by the method of Peterson & Sober (1956). Fractionation of crude leucocidin on this resin has been by gradient elution and by stepwise elution, buffers of increasing $[Na^+]$ being used at constant pH. A 0·4 M-acetate buffer, pH 5·0, was prepared by diluting 24 ml. of acetic acid and 13 g. of NaOH to 1000 ml. Buffers of lower concentration were made by dilution and buffers of higher $[Na^+]$ by addition of NaCl. In the experiments with gradient elution the $[Cl^-]$ in the effluent was determined by titration against $Hg(NO_3)_2$ with sodium nitroprusside as indicator (Kolthoff & Sandell, 1952).

Amberlite CG-50. This was regenerated and set up in columns by the method of Boardman & Partridge (1955). Citrate buffers were used with this resin: 0·1 M-citrate buffer, pH 5·0, contained 21 g. of citric acid, 8·07 g. of NaOH and 5·6 g. of NaCl/1000 ml.; 0·225 M-citrate buffer, pH 6·5, contained 47·7 g. of citric acid and 27·95 g. of NaOH/1000 ml.

Fractionation with ammonium sulphate. This was done by extracting the insoluble precipitates with $(NH_4)_2SO_4$ solutions of decreasing concentration.

Electrophoresis

Boundary electrophoresis. This was done in an Antweiler microelectrophoresis apparatus. The buffers were those described in the section on chromatography and also 0·1 M-

sodium carbonate buffer, pH 11·2, containing 10·6 g. of Na_2CO_3/1000 ml., was used. With protein concentration greater than 2% it was possible to compensate the boundary before applying the electric field by slowly withdrawing fluid from one of the electrode vessels.

Zone electrophoresis. The method of Kunkel & Slater (1952) for zone electrophoresis in slabs of starch grains was used. Zone electrophoresis in slabs of starch gel was by the method of Smithies (1955) except that the starch gel was made from a batch of potato starch (British Drug Houses Ltd.) which had been hydrolysed in acetone–HCl for 4·5 hr. Gels containing 15% of starch had satisfactory properties.

In each case dry starch grains were added to the solution of the starting material and the suspension was inserted in a slot 0·5 cm. wide. Glucose was inserted in the slab independently and its movement during electrophoresis taken as a measure of endosmotic flow. Its position at the end of the run was determined by placing a strip of filter paper on the slab and then staining it with a $AgNO_3$ reagent (Trevelyan, Procter & Harrison, 1950).

At the completion of the run the slabs were cut in 1 cm. sections which were eluted with 0·1 M-acetate buffer, pH 5·0. The sections from the starch gel were frozen and thawed before elution.

When the crude leucocidin in 0·1 M-acetate buffer, pH 5·0, was applied to a column of starch grains it appeared with the solvent front, which was taken as evidence that adsorption was slight.

Analytical methods

Nitrogen. This was determined by the micro-Kjeldahl method.

Dry-weight determinations. These were made on two samples of highly purified leucocidin, which were dialysed against distilled water, pH 5·0, for several days, centrifuged and the supernatants dried at 110° for 30 hr. The dry solid and weighing ampoule were then digested for the determinations of nitrogen.

Ultraviolet absorption. Measurements were made in a Unicam SP. 500 spectrophotometer.

Protein. Concentration of protein was assessed from the ultraviolet absorption on the assumption of an extinction coefficient $E^{0.1}_{280}$ % 1·0.

Tyrosine and tryptophan. These were estimated from the ultraviolet absorption by use of the formulae of Goodwin & Morton (1946). No corrections were made for scatter.

Immunological analysis. Diffusion in agar, by the method of Ouchterlony (1949), was used.

RESULTS

Development of a medium for production of leucocidin in high titre

The 'CCY' medium employed by Gladstone & van Heyningen (1957) gave a leucocidin titre of 1–2 L^+/ml. Difco Casamino acids could be replaced by the corresponding Oxoid product without affecting this result. The effect of some other modifications is shown in Table 1. Reduction of the glycerophosphate concentration decreased the yield and it could not be replaced by glycerol, nor could the lactate and glycerophosphate be replaced by glucose. Substitution of Oxoid yeast extract for the corresponding Difco product gave a tenfold increase in titre. The stimulating effect of Oxoid yeast extract was maximal at 20–25%, v/v; higher concentrations were inhibitory. Fractionation of the yeast extract on ion-exchange resins suggested that the stimulation of leucocidin production may be due to several substances acting in conjunction (A. M. Woodin, unpublished results).

Fractionation of the culture-filtrate slurry

Fractionation with ammonium sulphate. The culture-filtrate slurry was not completely soluble in 0·1 M-phosphate buffer, pH 6·7, and the solution was viscous and not suitable for application to the hydroxylapatite columns. The viscous component was absent in the fraction soluble in 1·2–2·2 M-$(NH_4)_2SO_4$, which contained 60–70% of the leucocidin, purified twofold.

This fraction was precipitated by saturation with $(NH_4)_2SO_4$. It contained diffusible substances absorbing strongly in the ultraviolet region and its protein content was assessed from the ultraviolet absorption of material which had been exhaustively dialysed against M-NaCl–0·1 M-phosphate buffer, pH 6·7.

Fractionation on hydroxylapatite. Preliminary experiments showed leucocidin to be adsorbed by hydroxylapatite at phosphate concentrations below 0·1 M and subsequently to be eluted when the concentration of phosphate was greater than 0·2 M.

When the product from the $(NH_4)_2SO_4$ fractionation was applied to a hydroxylapatite column a thin layer of very strongly adsorbed material was deposited on the surface. This clogging of the surface gradually decreased the flow rate and made the columns unstable. Channels frequently developed particularly when the pressure of the inflow was increased. This difficulty was overcome by dissolving the $(NH_4)_2SO_4$ product in 0·25 M-phosphate buffer, pH 6·7, and passing it through a small column of hydroxylapatite, the surface of which could be stirred from time to time. The clogging material was thus removed with complete recovery of leucocidin in the effluent, which was then diluted or dialysed to reduce the phosphate concentration to 0·1 M and applied to a fresh column of hydroxylapatite.

Fig. 1 shows the result of eluting from hydroxylapatite the crude leucocidin from the $(NH_4)_2SO_4$ fractionation. The leucocidin activity is eluted in more than one phosphate concentration range. This was not taken as evidence for fractionation of the material responsible for the activity, as the ability of a substance with a wide elution range to give rise to multiple peaks on stepwise elution from hydroxylapatite is known (Tiselius *et al.* 1956; Tiselius, 1958).

Table 1. *Effect of composition of the medium on leucocidin production by* Staphylococcus aureus

The Casamino acids and salts mixture was present in all media to give these final concentrations: Oxoid Casamino acids, 20 mg./ml.; $Na_2HPO_4,12H_2O$, 6·2 mg./ml.; KH_2PO_4, 0·4 mg./ml.; $FeSO_4,7H_2O$, 6·4 μg./ml.; citric acid, 6·4 μg./ml. $MnSO_4,4H_2O$, 10 μg./ml.; $MgSO_4,7H_2O$, 20 μg./ml. The pH was adjusted to 7·2–7·4 before inoculation. Cultures were in rocking T-tubes at 37° for 20 hr.

(*a*) *Effect of yeast diffusate*

Basic medium: Casamino acids and salts; sodium glycerophosphate, 20 mg./ml.; sodium lactate, 10 mg./ml.

Additions	Growth (mg. of organisms/ml.)	Leucocidin titre (L^+/ml.)
Bacto Yeast diffusate (20%, v/v)	10·5	1·25
Oxoid Yeast diffusate (20%, v/v)	8·2	14·0
Nicotinamide (2 μg./ml.) Thiamine (2 μg./ml.) Cystine (500 μg./ml.) Tryptophan (500 μg./ml.)	4·0	< 1·25

(*b*) *Effect of energy source*

Basic medium: Casamino acids and salts; Oxoid Yeast diffusate, 20%, v/v.

Additions	Concn. (mg./ml.)	Growth (mg. of organisms/ml.)	Leucocidin titre (L^+/ml.)
Sodium glycerophosphate Sodium lactate	40 20	7·0	11
Sodium glycerophosphate Sodium lactate	20 10	8·0	14
Sodium glycerophosphate Sodium lactate	10 5	9·0	8·8
Sodium glycerophosphate Sodium lactate	5 2·5	8·5	3·5
Glycerol Sodium lactate	14·5 10	6·2	3·4
Glycerol Glucose	14·5 10	3·5	<2·5
Sodium lactate Glucose	10 10	6·0 4·0	<2·5 <2·5

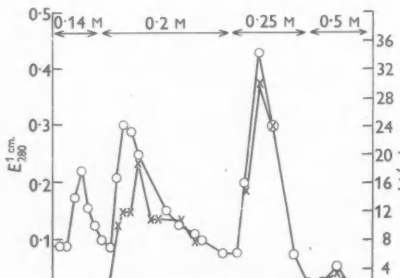

Fig. 1. Elution of crude leucocidin from a column of hydroxylapatite. A portion (142 mg.) of the $(NH_4)_2SO_4$ product from culture filtrate P_1, containing 2250 L^+ of leucocidin in 0·1 M-phosphate buffer, pH 6·7, was applied to a column 10 cm. × 1·4 cm. The horizontal arrows indicate the elution range during which the various phosphate buffers were applied to the column. ○, $E^{1\,cm.}_{280}$; ×, L^+/ml.

The amount of leucocidin eluted by 0·14 M-phosphate buffer was greater the smaller the column, indicating displacement effects. As extraneous protein was removed at this stage this small loss of leucocidin was not disadvantageous. In Fig. 1 there is little difference in the L^+/mg. of the effluent in 0·2, 0·25 and 0·5 M-phosphate buffer, pH 6·7, but with other preparations the 0·5 M-phosphate buffer effluent had less L^+/mg. than the 0·25 M-phosphate buffer effluent. As the purpose of the fractionation on hydroxylapatite was removal of extraneous protein, the columns were eluted with 0·14 M-phosphate buffer, pH 6·7, followed by 0·25 M-phosphate buffer, pH 6·7. This contained 40% of the leucocidin added to the columns purified two- to five-fold.

Concentration on carboxymethylcellulose. The eluent from hydroxylapatite in 0·25 M-phosphate buffer, pH 6·7, was adjusted to pH 5·0 with acetic acid and dialysed against 0·1 M-acetate buffer, pH 5·0. A small amount of precipitate was removed without loss of activity and the leucocidin concentrated by adsorption on a small column of

Table 2. *Properties of leucocidin preparations at various stages of purification*

With the exception of those marked with an asterisk all values are for exhaustively dialysed material.

Culture filtrate	P1		P4		P9	
Stage in purification	L^+/mg. of protein	E_{280}/E_{260}	L^+/mg. of protein	E_{280}/E_{260}	L^+/mg. of protein	E_{280}/E_{260}
Culture-filtrate slurry	7*	0·9*	19	1·22	10	1·20
$(NH_4)_2SO_4$ product	16*	1·05*	35	1·45	19	1·32
Effluent from hydroxylapatite in 0·25 M-phosphate buffer, pH 6·7	65	1·85	75	1·80	42	1·55
Product from concentration on carboxymethylcellulose	100	1·95	80	1·90	55	1·75

carboxymethylcellulose (3 ml. of adsorbent/g. of protein). Some inactive material was unadsorbed and rejected. The column was eluted with 2 M-acetate buffer, pH 5·0, with complete recovery of the leucocidin. For storage the solution was saturated with $(NH_4)_2SO_4$. This material will be known as the 'hydroxylapatite product'.

Comparison of different culture filtrates. Several 100 l. culture filtrates have been processed in the way described in the preceding paragraphs. The properties of the products from the various stages are given in Table 2, and it is clear that they are not identical. Nevertheless the fractionation achieved with $(NH_4)_2SO_4$ and on hydroxylapatite is similar in all cases, and when the effluent from the hydroxylapatite columns is concentrated on carboxymethylcellulose, further inactive material is unadsorbed in each case. The ratio E_{280}/E_{260} is an approximate measure of the nucleic acid content (Warburg & Christan, 1941), and reveals that nucleic acid is removed from each culture filtrate at each stage. The L^+/mg. is also increased in the fractionations listed in Table 2 and it is likely that this is mainly due to removal of inactive protein. It will be shown that the activity results from the synergistic action of two proteins and L^+/mg. values would also be increased by removal of that component which is present in excess. Separation of the component to such an extent that it was no longer in excess would be expected to result in loss of activity, and to decreased L^+/mg. values for protein, and so does not appear to occur at this stage.

Properties of the hydroxylapatite product

Stability. Solutions of the hydroxylapatite product in acetate buffers, pH 5·0, were stable at 2° for several days and so were solutions in phosphate buffers, pH 6·7, where the concentration was greater than 0·1 M. At lower salt concentrations at pH 6·7, however, irreversible precipitation of some of the protein occurred with some loss of activity. Similarly, dialysis against 0·1 M-borate buffer, pH 9·0, or 0·1 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer, pH 9·0, precipitated much of the protein with irreversible loss of some of the activity. The precipitate produced in this way, at pH 6·7 or 9·0, could be dissolved at pH 10·0 but without recovery of the activity. At pH 4·0, in 0·1 M-acetate buffer, solutions slowly acquired an opalescence and irreversibily lost activity.

Electrophoretic behaviour. Fig. 2 records the schlieren diagrams of the hydroxylapatite product migrating in an electric field. At pH 6·0 there was little migration of any of the components. Between pH 6·0 and 8·0 the components moved very slowly to the positive electrode. At pH 5·0 all the components were positively charged and there was considerable migration to the negative electrode. Particularly at pH 5·0 the ascending and descending boundaries were not identical and the appearance suggested the formation of reversibly dissociating complexes.

Table 3 gives the result of zone electrophoresis of the hydroxylapatite product from culture filtrate P 1 in a block of starch grains. The combining power was recovered over a wide zone; 91 % of the protein, but only 50 % of the combining power, was recovered. When the fractions were mixed the resulting solution had all the combining power of the starting material. A similar result was obtained with the hydroxylapatite product from culture filtrate P 2 on electrophoresis at pH 5·0 in a slab of starch gel. With this supporting medium complete recovery of the protein from the gel was not obtained. The L^+/mg. of the fractions was less than that of the starting material, but when they were mixed the L^+/mg. of the solution was identical with that of the starting material.

Fractionation of the hydroxylapatite product

Fractionation on Amberlite CG-50. Fig. 3 records the result of eluting the hydroxylapatite product from a column of Amberlite CG-50. When the solvent was changed from 0·1 M-citrate buffer, pH 5·0, to 0·225 M-citrate buffer, pH 6·5, a single

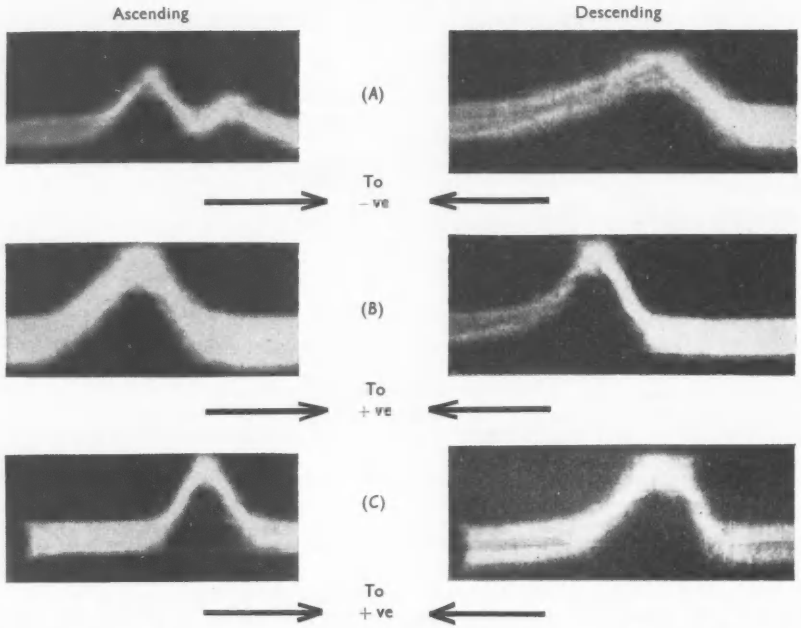

Fig. 2. Schlieren diagrams of the hydroxylapatite product from culture filtrate P9 migrating in an electric field. Protein concn., 2%. Ascending boundaries on the left-hand side. (*A*) 0·1 M-Acetate buffer, pH 5·0 (5 min. at 1·3 mA); (*B*) 0·1 M-phosphate buffer, pH 7·0 (18 min. at 2·0 mA); (*C*) 0·1 M-Na_2CO_3, pH 11·2 (8 min. at 2·5 mA). Starting positions at pH 5·0 and pH 11·2 were on the extreme left of the ascending-limb photographs. The boundary at pH 7·0 has moved very little.

Table 3. *Zone electrophoresis of hydroxylapatite product*

Hydroxylapatite product (42 mg.) from culture filtrate P1, containing 5000 L^+ of leucocidin, was added to a 0·5 cm. slot. Solvent was 0·1 M-acetate buffer, pH 5·0, current (35 mA) was passed for 20 hr. through a slab 7 cm. × 1 cm. × 30 cm. Migration was corrected for endosmosis.

Cathodic migration (cm.)	Protein recovered (%)	Leucocidin recovered (%)	Combining power of recovered material (L^+/mg. of protein)
4	6	2	42
5	22	10	58
6	43	32	95
7	20	6	80

trailing protein peak was observed in which the maximum combining power was not coincident with the maximum concentration of protein. The recovery of the combining power calculated from the individual volumes and combining powers of the fractions was only 20% of the input. However, when all the fractions were mixed and the mixture was assayed the recovery was quantitative. This finding, together with the electrophoretic heterogeneity, suggests that more than one substance is necessary for the activity and that they have been incompletely separated under these conditions. As it was known that the components differ little in electrophoretic mobility at pH 6·0, attempts to separate them by chromatography involving pH changes were abandoned in favour of chromatography at pH 5·0 with increasing salt concentration. On Amberlite CG-50 this is not feasible since all the components are retained by the resin even when the Na^+ concentration is 3·5 M.

Fractionation on carboxymethylcellulose. Fig. 4 records the result of eluting the hydroxylapatite product from carboxymethylcellulose with a salt gradient at pH 5·0. Again there is separation into relatively more and relatively less active fractions but recovery was only quantitative when the fractions were mixed. Further evidence that more than one substance is required for the activity was

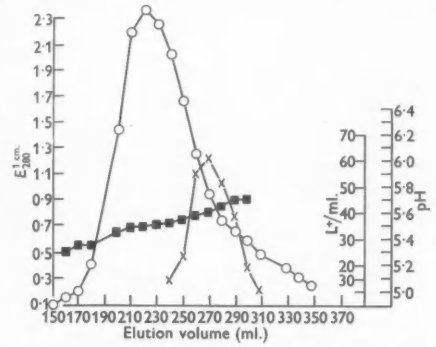

Fig. 3. Elution of the hydroxylapatite product from Amberlite CG-50. A portion (252 mg.) of the hydroxylapatite product from culture filtrate P9, containing 18 000 L^+ of leucocidin, in 0·1 M-citrate buffer, pH 5·0, was adsorbed on a column 11 cm. × 1·4 cm. Elution volume was measured after changing inflow buffer to 0·225 M-citrate buffer, pH 6·5. ○, $E^{1\,cm.}_{280}$; ■, pH; ×, leucocidin titre.

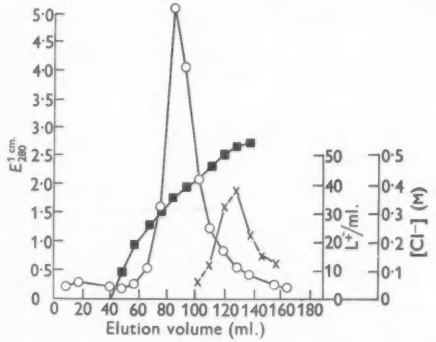

Fig. 4. Elution of the hydroxylapatite product of culture filtrate P9 from carboxymethylcellulose with a salt gradient. A portion (180 mg.) containing 6600 L^+ of leucocidin was added to a column 40 cm. × 0·6 cm. Gradient elution was from 0·1 M-acetate buffer, pH 5·0, to M-NaCl–0·1 M-acetate buffer, pH 5·0. ○, $E^{1\,cm.}_{280}$; ■, $[Cl^-]$; ×, L^+/ml.

obtained in the following way. In continuation of the experiment recorded in Fig. 4 the fractions eluted in the volume 66–100 ml. and those in the volume 120–190 ml. respectively were combined (i.e. those fractions eluted in the volume 100–120 ml. were omitted). These two solutions were assayed separately and then mixed together. The result, recorded in Table 4, shows clearly that there is synergism of the two fractions when they are mixed. There is little significant difference between the MLeD/L^+ ratio of each fraction and their mixture, which is evidence that little formation of toxoid occurs.

At lower concentrations of the faster-moving components of the hydroxylapatite product better chromatographic separation was obtained. A sample of the hydroxylapatite product was adsorbed on a column of carboxymethylcellulose and fractions were separated by stepwise addition of solvents of increasing salt concentration. The results are recorded in Table 5. Only the last fraction had significant leucocidin activity, representing 30 % of the input, and was adsorbed on a further column of carboxymethylcellulose and eluted with a salt gradient at pH 5·0. Fig. 5 records the result and shows a clear separation into two protein peaks.

These results suggested that the components of the hydroxylapatite product could be separated by a two-stage stepwise elution followed by a gradient analysis on carboxymethylcellulose. A portion (700 mg.) of the hydroxylapatite product from culture filtrate P9 was applied to a column of 5 g. of carboxymethylcellulose and the following fractions were obtained: (1) that eluted by 0·175 M-NaCl–0·2 mM-acetate buffer, pH 5·0; (2) that eluted by 2 M-acetate buffer, pH 5·0. Then 100 mg. of (2) was adsorbed on a further carboxymethylcellulose column and eluted with a salt gradient which gave two protein peaks (Fig. 5). The two fractions eluted by stepwise elution will be referred to as displacement eluates 1 and 2. The two obtained by further fractionation of (2) by gradient elution will be referred to as the fast and slow fractions. The displacement eluate 1 and the fast and slow fractions from this experiment were each re-chromatographed on the same carboxymethylcellulose column with a salt gradient. The results of

Table 4. *Combining power of the fractions of the hydroxylapatite product*

The fractions were separated on carboxymethylcellulose in the way described in Fig. 5.

Elution ranges (ml.)		Volume (ml.)	L^+/mg. of protein	Leucocidin yield (L^+/ml. × volume)	MLeD/L^+
66–100		35	1·9	150	1000
120–190		70	80	1900	300
66–100 + 120–190	Mixed	Calc. 105	52	2050	560
		Found 105	42	4200	400

these three experiments show (Fig. 6) that each protein peak emerged in the same salt concentration range by which it had originally been separated. Only the slow fraction had sufficient activity to permit measurement of the test dose and these measurements suggest that this residual activity is a consequence of contamination with a small amount of the fast fraction. The fractions from re-chromatography of displacement eluate 1 and of the fast fraction were each assayed in the presence of a constant excess of the slow fraction. These assays indicate slight heterogeneity of the fast fraction and considerable heterogeneity of displacement eluate 1.

That the poor separation of the fast and slow fractions in direct-gradient analysis of the hydroxylapatite product is due to overlap of the fractions and not to their interaction was confirmed in the following way. The hydroxylapatite product from

Table 5. *Elution of the hydroxylapatite product from carboxymethylcellulose by stepwise increases in concentration of salt*

The product (210 mg.), containing 12 000 L^+ of leucocidin, was applied to a column 2·4 cm. × 5·0 cm.

Eluent	Total protein eluted (%)	L^+/mg. of protein
0·2 M-Sodium acetate, pH 5·0 0·042 M-NaCl	6·5	1·15
0·2 M-Sodium acetate, pH 5·0 0·07 M-NaCl	12·5	1·0
0·2 M-Sodium acetate, pH 5·0 0·175 M-NaCl	51	5·2
0·2 M-Sodium acetate, pH 5·0 0·58 M-NaCl	22	58·0

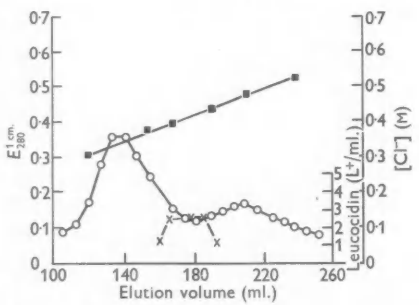

Fig. 5. Gradient elution of partially purified leucocidin from carboxymethylcellulose, demonstrating the separation into fast- and slow-moving components. A portion (26 mg.) containing 1500 L^+ was applied to a column 35 cm. × 1·0 cm. The gradient was from 0·1 M-acetate buffer, pH 5·0, to M-NaCl–0·1 M-acetate buffer, pH 5·0. ○, $E^{1\,cm.}_{280}$; ×, L^+/ml.; ■, [Cl^-].

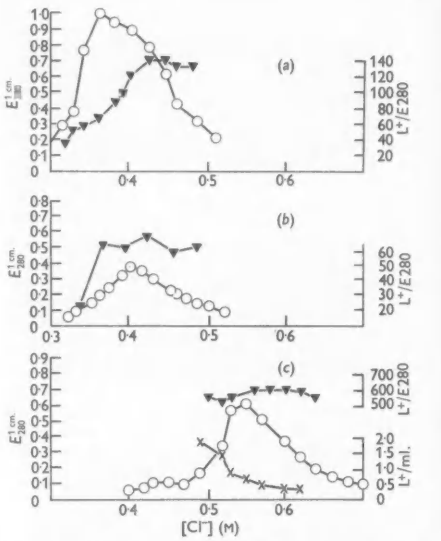

Fig. 6. Rechromatography on carboxymethylcellulose of the three fractions derived from the hydroxylapatite product. The same column of carboxymethylcellulose was used for each component with the rejection of the top 2–3 mm. of resin between each experiment. Column dimension was 35 cm. × 1·0 cm. (*a*) First displacement eluate. (*b*) Fast fraction. (*c*) Slow fraction. ○, $E^{1\,cm.}_{280}$; ▼, L^+ determination in the presence of second fraction (see text); ×, L^+ determinations without additions.

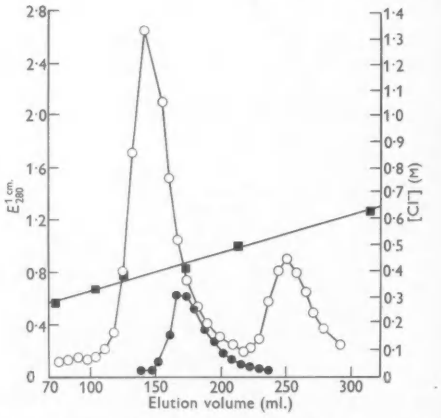

Fig. 7. Gradient elution of the hydroxylapatite product from culture filtrate P1 and rechromatography of the fast fraction after displacement separation (see text). The same column (39 cm. × 1 cm.) was used for each gradient analysis. ■, [Cl^-]; ○, $E^{1\,cm.}_{280}$ for hydroxylapatite product; ●, $E^{1\,cm.}_{280}$ for rechromatography of fast fraction.

culture filtrate P1 was fractionated by gradient elution on carboxymethylcellulose and the material eluted in the range 0·335–0·490 M-Cl^- was adsorbed on a further column of carboxymethylcellulose and separated into two fractions by stepwise addition of 0·175 M-NaCl–0·2 M-acetate buffer, pH 5·0, followed by 2 M-acetate buffer, pH 5·0. The latter fraction was adsorbed on the same carboxymethylcellulose column used for the gradient analysis of the hydroxylapatite product and eluted with a salt gradient. There is no evidence for the appearance of two peaks and little material is eluted above 0·49 M-Cl^- (Fig. 7).

Gradient analysis of different batches of the hydroxylapatite products

Gradient analysis of the hydroxylapatite products has shown only two components but the proportions of these have differed. Culture filtrates P1, P4 and P9 have given hydroxylapatite products with L^+/mg. of 100, 80 and 55 respectively (Table 2), and these contained 25, 13 and 4·5 % of the slow fraction. It is possible that the different L^+/mg. values are a consequence of the different ratios of the fractions but correlation must await a more precise determination of the contribution of each fraction to the total L^+ value.

Properties of the components of the hydroxylapatite product

On the basis of the preceding experiments the procedure described in Table 6 was used to prepare the displacement eluate 1 and the fast and slow fractions of leucocidin. They were stored in saturated $(NH_4)_2SO_4$.

The slow fraction of leucocidin dissolved readily in 0·1 M-acetate buffer, pH 5·0, or in 0·2 M-phosphate buffer, pH 7·0. Solutions of 3 % (w/v) concentration were clear, free from pigment and were stable for several days at 2°; on dialysis against distilled water they remained clear. Boundary electrophoresis in 0·1 M-acetate buffer, pH 5·0, or in 0·2 M-phosphate buffer, pH 7·0, showed a single component. At pH 5·0 the slow component is positively charged, at pH 7·0 it is negatively charged but migrates very slowly. Immunological analysis by the Ouchterlony method showed two bands at 0·4 % antigen concentration, of which one showed a reaction of identity with one of the bands of the fast fraction of leucocidin and was not visible at 0·1 % antigen concentration (Fig. 8). Comparison of the immunological analysis of two-fold dilutions of the slow and fast fractions of leucocidin indicated that the slow fraction was contaminated with about 3 % of the fast fraction. The slow fraction contained 15·0 % of N, 8·2 % of tyrosine, 2·2 % of tryptophan and the ratio E_{280}/E_{260} was 2·15 at pH 5·0, indicating absence of nucleic acid.

The fast fraction was dissolved in 0·1 M-acetate buffer, pH 5·0, and gave solutions which at 3·5 % were slightly brown. On standing these solutions developed an opalescence and in several days deposited a precipitate. Boundary electrophoresis in 0·1 M-acetate buffer, pH 5·0, showed two peaks in the ascending limb with fusion of the two peaks in the descending limb. The schlieren diagram was similar to that of the hydroxylapatite product from culture filtrate P9 (Fig. 2). The precipitate stored in saturated $(NH_4)_2SO_4$ dissolved in 0·2 M-phosphate buffer, pH 7·0, but on dialysis against this solvent a precipitate was deposited containing 40 % of the total material. The supernatant on boundary electrophoresis at pH 7·0 showed a single peak migrating slowly to the anode. Immunological analysis by the Ouchterlony method showed three bands, a sharp band nearest the antibody well and two diffuse bands. At 0·2 % antigen concentration the diffuse bands fused. As the antigen was successively diluted the outermost diffuse band appeared increasingly nearer the antigen well but the position of the other bands was little affected (Fig. 8). The fast fraction contained 15·2 % of N, 8·4 % of tyrosine and 2·7 % of tryptophan. The ratio E_{280}/E_{260} was 1·95 at pH 5·0, indicating that little nucleic acid was present.

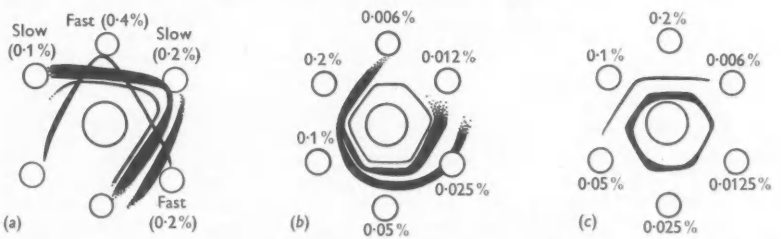

Fig. 8. Immunological analysis of the fast and slow fractions of leucocidin. In all cases the antiserum was in the centre well. (*a*) Fast and slow fractions to show the reaction of identity of the contaminant of the slow fraction with one of the bands of the fast fraction. (*b*) Serially diluted fast fraction. (*c*) Serially diluted slow fraction.

Table 6. *Fractionation of culture filtrate P 12*

P12 contained 1 300 000 L^+ of leucocidin.

CULTURE-FILTRATE SLURRY (75 g. of protein; 1 050 000 L^+)

Extracted with 2·2 M-$(NH_4)_2SO_4$

Solution rejected

PRECIPITATE

Extracted with 1·2 M-$(NH_4)_2SO_4$

Precipitate rejected

SOLUTION [700 000 L^+; 22·5 g. of protein]

Dialysed against 0·25 M-phosphate buffer, pH 6·7

Diffusate rejected

DIALYSED SOLUTION

Passed down hydroxylapatite column

Adsorbed material rejected

EFFLUENT SOLUTION

Dialysed against 0·1 M-phosphate buffer, pH 6·7

Diffusate rejected

DIALYSED SOLUTION

Passed down hydroxylapatite column

Effluent rejected

ADSORBED MATERIAL ON HYDROXYLAPATITE

Eluted with 0·14 M-phosphate buffer, pH 6·7

Effluent rejected

ADSORBED MATERIAL ON HYDROXYLAPATITE

Eluted with 0·25 M-phosphate buffer, pH 6·7

Adsorbed material rejected

EFFLUENT SOLUTION [360 000 L^+; 5·2 g. of protein]

Dialysed against 0·1 M-acetate buffer, pH 5·0

Diffusate and precipitate rejected

DIALYSED SOLUTION

Passed down column of carboxymethyl-cellulose

Effluent rejected

ADSORBED MATERIAL ON CARBOXYMETHYLCELLULOSE

Eluted with 0·175 M-NaCl, 0·2 M-acetate buffer, pH 5·0

Displacement eluate 1 (3·09 g. of protein)

Table 6 (*cont.*)

ADSORBED MATERIAL ON CARBOXYMETHYL-CELLULOSE

Eluted with 2 M-acetate buffer, pH 5·0

Adsorbed material rejected

EFFLUENT SOLUTION (590 mg. of protein)
DISPLACEMENT ELUATE 2

Diluted to give solution in 0·1 M-acetate buffer, pH 5·0

SOLUTION

Passed down column of carboxymethyl-cellulose

Effluent rejected

ADSORBED MATERIAL ON CARBOXYMETHYL-CELLULOSE

Gradient applied to M-NaCl, 0·1 M-acetate buffer, pH 5·0

FAST FRACTION (80 mg. of protein) | SLOW FRACTION (380 mg. of protein)

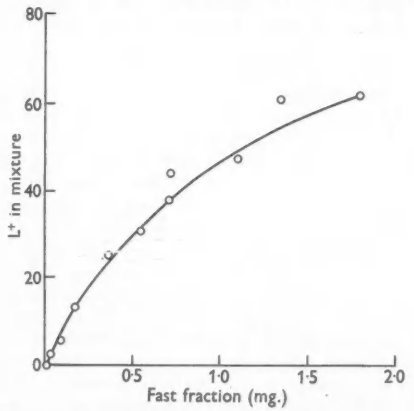

Fig. 9. Effect on the anti-leucocidin-combining power of addition of the fast fraction to a constant amount of the slow fraction. Assay was by the combining-power method. The mixtures contained 0·076 mg. of slow fraction.

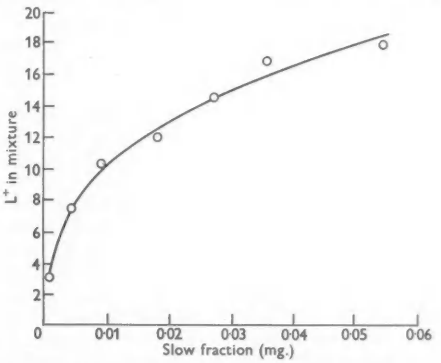

Fig. 10. Effect on the anti-leucocidin-combining power of addition of the slow fraction to a constant amount of the fast fraction. Assay was by the combining-power method. All mixtures contained 0·22 mg. of fast fraction.

The displacement eluate 1 has not been studied in any detail. The precipitate stored in saturated $(NH_4)_2SO_4$ was brown. Boundary electrophoresis in 0·1 M-acetate buffer, pH 5·0, showed two peaks in the ascending limb and these were fused in the descending limb in the way characteristic of the hydroxylapatite product of culture filtrate P 9 (Fig. 2). Immunological analysis by the Ouchterlony method revealed that in addition to the three bands characteristic of the fast fraction of leucocidin other antigens were present in solutions more concentrated than 0·2 %.

Properties of mixtures of the fast and slow fractions

Boundary electrophoresis of mixtures of the fast and slow fractions in 0·2 M-phosphate buffer, pH 7·0, showed a single peak, but as the mobilities of the isolated components are similar at this pH value, this cannot be taken as evidence for complex formation.

Figs. 9 and 10 show the effect of varying amounts of each fraction in the presence of a constant

Table 7. *Leucocidin activity of a mixture of the slow and fast fractions*

All mixtures contain 1 μg. of slow fraction. Assay was by comparison of the activities of twofold dilutions.

Fast fraction (μg.)	0	2·0	4·8	5·8	10	20	40
MLeD in mixture	20	400	800	1600	2400	2400	2400

amount of the other on the leucocidin titre determined by the combining-power method. Table 7 shows the effect of varying amounts of the fast fraction with a constant amount of the slow fraction on the MLeD titre. Ouchterlony plates developed with mixtures of the fast and slow fractions in the antigen wells had the appearance expected from independent development of the bands characteristic of the isolated fractions.

DISCUSSION

By methods depending upon the molecular charge it has been possible to separate leucocidin into two main fractions. The separation is accompanied by loss of activity, which is regained by mixing the fractions. It has not been possible to prepare either of the two fractions completely free from residual activity but there is evidence that the little activity each has is a consequence of contamination with the other.

The two fractions have a nitrogen content, amphoteric behaviour and ultraviolet absorption consistent with their containing little material other than protein. One of them, the slow fraction, behaves immunologically, electrophoretically and chromatographically as a single substance (except for contamination with the fast fraction). However, it is well known that proteins trail considerably on hydroxylapatite and on carboxymethylcellulose, and it is not considered that either fraction of leucocidin is quite free from trace contamination with other products of the *Staphylococcus*. It is probable that an antiserum prepared against either fraction would contain antibodies to other materials.

The other fraction of leucocidin, apart from the possibility of trace contamination, does not behave as a single substance. Immunological analysis by diffusion in agar shows three bands, of which two persist to small antigen concentrations, and electrophoresis at pH 5·0 shows, in the ascending limb, clear separation of two boundaries. In the descending limb these are less clearly defined and there appears to be a third component separating the regions corresponding to the two major components. This asymmetry can arise in boundary electrophoresis from the partial separation of two components which interact to form a complex. Such interaction between particles of different net charge could account for the failure of the components of the fast fraction to separate on the cation-exchange resins.

It is not known whether all of the components in the fast fraction are necessary, together with the slow fraction, to give leucocidin activity. It is also not known if there is interaction between the slow and fast fractions at pH 7·0. As precipitin bands corresponding to the isolated fractions are produced by mixtures it is clear that the interaction, if it exists, is weak and that some of the free fractions are also present. Moreover, separate antibodies to each are present in staphylococcal antitoxin, suggesting that in the bacterial filtrate the two are not in a complex. It is therefore possible that the death of the white blood cell induced by leucocidin is due to the two components acting completely independently, the action of each, alone, being such that the morphological changes described by Gladstone & van Heyningen (1957) or the inhibition of respiration are not brought about.

Mixtures of the fast and slow fractions have a maximum activity when the ratio of the former to the latter is about 10:1. This figure is subject to the lack of precision of the activity assay. The more precise combining-power method gives an ambiguous result. The shape of the curves in Figs. 9 and 10 does not permit calculation of the ratio of fast to slow at maximum combining power, but if the ratios are compared for (maximum combining power)/2, Fig. 9 gives a value of 9 and Fig. 10 a value of 27·5. This divergence is not unexpected, for the antiserum contains antibodies to both components and the antibody-combining-power method of assay would give a meaningful value for the optimum ratio of the components only if there were a stable complex formed which was responsible for the activity, and if the antiserum contained a specific antibody to this complex. There is no evidence that this is the case. It also follows from these considerations that the antibody-combining-power method of leucocidin assay must be treated with caution. It has been shown that a particular L^+ value can result from mixtures of quite different composition. Similarly it is impossible to set up a standard antitoxin unit, for different antisera may contain different proportions of the different antibodies.

When the work described in this paper was started it was assumed that there was a single substance responsible for the leucocidin activity. It is not now considered that the method used to

isolate the two fractions of leucocidin is necessarily the most efficient. Many other fractionation procedures than those described in this paper were tried but gave poor recoveries of the activity. It is now clear that this may have been due to separation of the fractions and not to irreversible inactivation. Nevertheless the method of purification described here permits the production of highly purified materials in reasonable yield and these are suitable for assessing the significance of leucocidin in staphylococcal pathogenicity. The separation of leucocidin into two fractions will permit controlling of such biological experiments; any effect possessed by one component, if it is due to an action on the white blood cell, must be multiplied manyfold by addition of the other.

SUMMARY

1. A medium suitable for large-scale production of leucocidin in high titre by *Staphylococcus aureus* has been developed.

2. An assay method for leucocidin suitable for routine use, based on the ability of leucocidin to prevent reduction of phenol-indophenol-2:6-dichlorophenol by macrophages has been developed.

3. Crude leucocidin can be partially purified by fractionation with ammonium sulphate and on hydroxylapatite.

4. On columns of cation-exchange resins leucocidin separates into two main fractions. There is loss of activity during the separation which is regained by mixing the fractions.

5. The nitrogen contents, ultraviolet-absorption and amphoteric behaviour of the fractions of leucocidin are characteristic of proteins.

I am greatly indebted to Dr D. W. Henderson of the Microbiological Research Establishment, Porton, for providing the facilities for the large-scale production of the culture filtrates; to Mr R. Elsworth and Mr R. Telling for carrying this out; to Miss Mollie Barr of the Wellcome Research Laboratories for the staphylococcal antitoxin; to Dr W. E. van Heyningen for his valued criticism and advice; and to Miss P. Womar and Mr L. Taylor for their technical assistance.

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The Starch of the Tuber and Shoots of the Sprouting Potato

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Relatively few chemical investigations have been carried out on the amylose content of the potato-starch granule during growth (see Greenwood, 1956). Meyer & Heinrich (1942) isolated starch from the leaves, shoots and tubers and reported the amylose contents to be 22, 73 and 37 % respectively. The percentage of amylose determined by potentiometric iodine titration of the starch in the growing tuber was shown by Halsall, Hirst, Jones & Sansome (1948) to remain constant at 17 % for two varieties. These authors also found the average length of unit chain of the amylopectin components (calculated from periodate oxidation results on the whole starch) to be constant at 24–26 anhydroglucose units. There is, however, evidence that the percentage of amylose may vary with the botanical variety of the tuber. Anderson & Greenwood (1955) have found small variations in iodine affinity of

3·94–4·03 %, representing changes of 20–21 % of amylose, and Doremus, Crenshaw & Thurber (1951) have reported variations of 17·5–21·7 % in 22 varieties of sweet potato.

In the work reported here we have studied the physical and chemical characteristics of the starch isolated from the shoots and tubers of the sprouting potato in comparison with those of the original tuber starch.

EXPERIMENTAL

Isolation of starches. Starch was isolated immediately after harvesting the tubers (var. Home Guard) by extraction with 0·01 M-$HgCl_2$ (to inhibit enzymic activity), sedimentation of the granules after filtration and deproteinization by shaking with toluene and 4% NaCl soln. as previously described (Cowie & Greenwood, 1957*a*). Some tubers were then kept at room temperature in semi-darkness until sprouts of up to 3 in. in length were formed. These were removed and the starch in them was isolated as described above. The residual flaccid tubers were similarly extracted. Although no quantitative estimate was made, the yield of starch from all three sources was high.

Estimation of granular size distribution. Size distributions were obtained by enlarging photomicrographs (four or five fields per sample) to give a final magnification of about 350 diameters and by measuring with a rule (to the nearest millimetre) the size of at least 500 granules for each starch. In view of the ellipsoidal nature of potato-starch granules, each was characterized by the length of its major axis (d). Number-average and weight-average particle diameters were calculated from $\Sigma n_i d_i / \Sigma n_i$ and $\Sigma n_i d_i^4 / \Sigma n_i d_i^3$ respectively, where n_i is the number of granules of diameter d_i. (In the latter case, this assumes that the ratio major:minor axis was constant for all granules.)

Gelatinization temperatures. The number of granules gelatinized at a particular temperature in water was determined with a microscope and Kofler electrically-heated microscope stage as described by Schoch & Maywald (1956).

Determination of phosphorus. Percentages of phosphorus in the starches and their components were determined by wet oxidation of the polysaccharide with perchloric acid (Smith, 1953), followed by colorimetric estimation of the phosphomolybdate complex (Fogg & Wilkinson, 1958). Weights of polysaccharide taken were: amylose, 500 mg.; starch, 150 mg.; amylopectin, 100 mg. Results were reproducible to ± 5% for the starch and amylopectin and to ± 15% for the amylose.

Aqueous leaching and fractionation. Portions of the starches were leached with water at 60° for 10 min. under N_2 and the amylose was recovered as the butan-1-ol complex. Fractionation was achieved by complete dispersion of the starch in water by boiling for 1 hr. under N_2, followed by the addition of thymol to precipitate the amylose. The amylose was purified by reprecipitation as the butan-1-ol complex. These methods have been given in detail elsewhere (Cowie & Greenwood, 1957*b*).

Characterization of the fractionation products. The purity of the amylose and amylopectin components was obtained by potentiometric iodine titration with a semi-micro differential apparatus (Anderson & Greenwood, 1955). Titration conditions were: [I′], 0·01 M; [total free I_2]$_{max.}$, 10 μM; temperature, 20°. Limiting viscosity numbers $[\eta]$ were obtained graphically from $[\eta] = \lim_{c \to 0} \eta_{sp.}/c$, where $\eta_{sp.}$ is the specific viscosity; concentrations (c) are expressed in g./ml. Measurements were made on the components dissolved in M-KOH at 22·5° with a modified Ubbelohde viscometer; detailed procedures are given by Cowie & Greenwood (1957*a*). A Spinco model E analytical ultracentrifuge was used to obtain the sedimentation constants (S_{20}) of the amylopectin components dissolved in water. The procedure and methods of evaluating the data have been described by Greenwood & Das Gupta (1958). Number-average degrees of polymerization for the amylose fractions were calculated from the relation: number-average degree of polymerization = 7·4 × $[\eta]$ (Cowie & Greenwood, 1957*b*). The percentage conversion of samples of both amylose and amylopectin into maltose under the action of purified soya-bean β-amylase (Peat, Pirt & Whelan, 1952) was determined as described previously; the enzyme was free from maltase and α-amylase activity and contained no Z-enzyme (Bryce, Cowie, Greenwood & Jones, 1958). [Z-Enzyme (see Peat, Thomas & Whelan, 1952) is capable of effectively removing barriers to β-amylolysis found in amylose.] Average lengths of unit chain for the amylopectins were calculated from the results of a potentiometric estimation of the formic acid released on oxidation with $NaIO_4$ at 2° (Potter & Hassid, 1948).

RESULTS AND DISCUSSION

Physical characteristics of the starches

Potentiometric-titration experiments indicated small but reproducible differences between the iodine affinity of the different starches. Corresponding percentages of amylose are shown in Table 1. The starch from the shoots with 16·7 % of amylose differs significantly from the other two.

Table 1. *Physical properties of the starches*

Average gelatinization temperature is the temperature at which 50 % of the granules are gelatinized.

Starch source	Iodine affinity (mg. of I_2 bound/100 mg. of starch)	Amylose* (%)	Av. granular diam. (μ) No.	Av. granular diam. (μ) Wt.	Av. gelatinization temp.	Phosphorus (%)
Original tuber	3·94	20·2	42	55	61°	0·093
Shoots	3·25	16·7	14	19	61·5	0·071
Sprouted tuber	3·96	20·3	40	51	62·5	0·071

* Calculated from [(iodine affinity ÷ 19·5) × 100]; Cowie & Greenwood (1957*b*).

Fig. 1 shows the number- and weight-percentage distribution curves for the granules. Calculated averages are given in Table 1. The two tuber starches possess granules of similar size and differ only in that the proportion of large granules (i.e. $>80\,\mu$) is greater in the unsprouted tuber starch. This difference is emphasized in the weight-distribution curves. The size–distribution curves for the shoot starch are completely different, the average granular size being only about a third of that for both tuber starches. This suggests that the shoot starch may be immature.

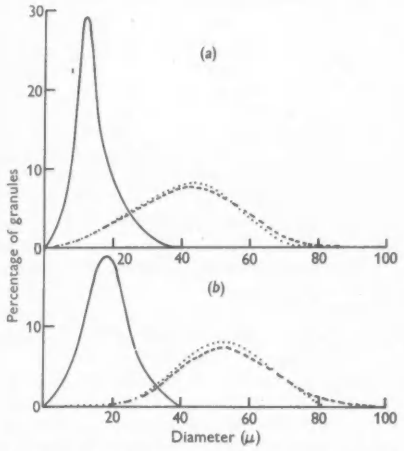

Fig. 1. Size–distribution curves for (*a*) number percentage and (*b*) weight percentage of granules. – – –, Starch from original tubers; —, starch from shoots; ..., starch from sprouted tubers.

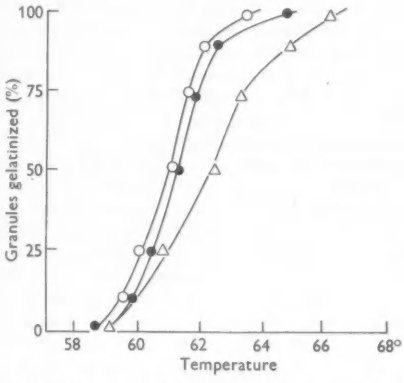

Fig. 2. Percentage of granules gelatinized in water as a function of temperature. ○, Starch from original tubers; ●, starch from shoots; △, starch from sprouted tubers.

Gelatinization curves are shown in Fig. 2, and the average gelatinization temperatures are given in Table 1. The curves are typical and illustrate the range of about 5° over which gelatinization of a starch occurs. The differences observed between the starches are again reproducible but are small and not of obvious significance. Granule size, the percentage of amylose present, the molecular weight of the components and the corresponding micellar organization of the granule may all influence gelatinization. An increase in amylose content raises the average gelatinization temperature (Savage, Deatherage, MacMasters & Senti, 1958). On the other hand, granular size is also important, as in any experiment large granules are seen to gelatinize first, whereas a few small ungelatinized granules often remain when the process is otherwise complete. For the two tuber starches, which have the same amount of amylose, the effect of the very large granules in the original starch may account for the differences in average gelatinization temperature. Although the shoot starch contains less amylose, the granular size is small. The observed gelatinization temperature must be accounted for by the interplay of these two effects, although the whole phenomenon is very complex.

The phosphate content of the original tuber starch appears to be higher than that for the other two starches. [Phosphorus occurs in potato starch as phosphate on C_6 of the glucose unit (Posternak, 1950).]

Aqueous leaching and fractionation

Our previous work (Cowie & Greenwood, 1957*b*) has shown that when potato starch of different botanical varieties is dispersed and fractionated under careful oxygen-free conditions, an amylose with a degree of polymerization of 3000–4000 is obtained. Further, aqueous leaching at 60–70° results in the preferential extraction of an amylose fraction of relatively low number-average degree of polymerization (~ 2000). Table 2 shows that the original starch gave results in agreement with this, which supports the suggestion of Cowie & Greenwood (1957*b*) that the molecular size of amylose is to some extent independent of the botanical variety of potato. Essentially similar results were obtained for the starch from the sprouted tuber. Although these tubers had obviously undergone great changes in physical state, surprisingly little enzymic modification or degradation of the granular starch has occurred. However, the number-average degree of polymerization of the aqueous-leached and total amyloses from the shoot starch are both significantly smaller, whereas the percentage conversion of the total amylose into

Table 2. *Properties of the fractions obtained from aqueous leaching and dispersion of the starches*

Granules were leached with water at 60° for 10 min., or dispersed in water at 100° for 1 hr.; both experiments were carried out in N_2.

	Original tuber		Shoot		Sprouted tuber	
	Leach	Dispersion	Leach	Dispersion	Leach	Dispersion
Amylose						
Percentage of total amylose*	45	>95	45	>95	40	>95
Purity (%)*	>98	>98	—	>98	—	>98
Phosphorus (%)	—	0·005	—	0·001	—	0·004
$[\eta]$ (g./ml.)	270	450	210	315	270	435
Degree of polymerization	2000	3300	1600	2300	2000	3200
β-Limit†	—	83	—	94	—	83
Amylopectin						
Purity (%)*	89	99·5	89	99·5	88	99·0
Phosphorus (%)	—	0·111	—	0·089	—	0·097
$[\eta]$ (g./ml.)	—	160	—	160	—	160
β-Limit†	—	56	—	56	—	57
Chain length	—	24	—	24	—	24
External chain length‡	—	16	—	16	—	16

* Calculated from iodine titrations. † Percentage conversion into maltose on β-amylolysis.
‡ Calculated from [(chain length × β-limit) + 2·5] (Peat, Whelan & Thomas, 1952).

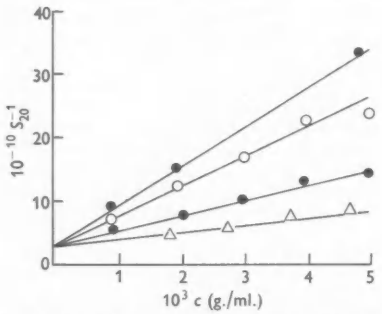

Fig. 3. Relation between the inverse of the sedimentation coefficient (S_{20}^{-1}) and the concentration (c) for the amylopectins dissolved in water. ○, Amylopectin from original tuber starch; ●, independent runs on amylopectin from shoot starch (see text); △, amylopectin from sprouted-tuber starch.

maltose on β-amylolysis is very much higher. The amount of phosphorus in this amylose was significantly lower.

On dispersion and fractionation the amylopectins obtained from all the starches were identical within experimental error in phosphorus content, average length of unit chain, percentage conversion into maltose with β-amylase and calculated average exterior chain lengths (see Table 2). As far as could be determined there was also little difference in the molecular size. Measurements of the molecular size of amylopectin are difficult (see Greenwood, 1956). Although the limiting viscosity numbers were identical, this is not conclusive as viscosity measurements are not particularly sensitive for detecting small changes in size for such highly branched molecules. Sedimentation measurements are more satisfactory in theory, but are complicated by the high concentration-dependence observed for the sedimentation coefficient (Greenwood & Das Gupta, 1958). Previously, we have made the measurements in alkaline solution. Here we have studied aqueous solutions, and the results of typical measurements are shown in Fig. 3. In agreement with Erlander & French (1958) it was found that the graph S_{20}^{-1} versus c (where S_{20} is the sedimentation coefficient corrected to water at 20°) was linear and allowed extrapolation to infinite dilution. However, the accuracy of such an extrapolation is limited in view of the high values of S_{20} involved. It was found that independent runs on the same sample gave curves with the same intercept but different slopes (see Fig. 3). This is thought to be due to small variations in the salt content of the solvent altering the ionization of the phosphate groups and so influencing intermolecular entanglements. Sedimentation in 0·2 M-NaCl was unsuccessful as aggregation occurred as shown by the presence of two or more sedimenting peaks on the schlieren pattern. This agrees with the light-scattering experiments of Witnauer, Senti & Stern (1955), who observed aggregation in the presence of salt of concentrations greater than mM. These authors suggested that the salt destroyed the repulsion between the ionized phosphate groups.

The problem of the ultracentrifuging of amylopectin is complicated, and only a limited number of investigations have yet appeared in the literature (Greenwood & Das Gupta, 1958; Erlander & French, 1958; Stacy & Foster, 1957). Further studies are being made. At most, it can be said

that S_{20} for the potato amylopectins studied here in aqueous solution is large and probably at least 500×10^{-13} c.g.s. units.

Utilization of the starch granule

The similarity in physical and chemical properties of both tuber starches suggests that when granules are utilized enzymically they are individually completely broken down to soluble material rather than being partially attacked. Furthermore, the extremely large granules may well be utilized first, a fact which may be related to their looser organization as shown by gelatinization studies. Both starches contain about 20% of amylose. This value differs radically from that of 37% found by Meyer & Heinrich (1942). However, these authors did not isolate whole granules and their procedure would preferentially extract amylose from the plant tissues.

Nature of the barrier to β-amylolysis

The starch from the shoots contained 16% of the linear component, in contrast to the 73% reported by Meyer & Heinrich (1942). As the starch is unlikely to have been transported to the shoots in granular form, the possibility exists that it may be immature. Also, the molecular size of the corresponding amylose is considerably smaller than for the tuber starches and the barrier to β-amylolysis (see, Peat, Pirt & Whelan, 1952) is more limited. The nature of the barrier to β-amylolysis is uncertain. It may be an artifact introduced during the isolation of fractionation of the starch (see, Baum, Gilbert & Scott, 1956). Equally well it may be native to the amylose. In the latter case the possibility of phosphorus giving rise to the barrier must be considered (compare, however, Peat, Thomas & Whelan, 1952). The amount of phosphorus in all the amyloses is very small (see Table 2) and consequently its estimation is difficult. Also, the presence of an amylopectin impurity of about 1% would cause a considerable error in the given phosphorus contents. Nevertheless, it is thought significant that the shoot amylose, which has the highest conversion into maltose on β-amylolysis, should have the lowest phosphate content. The problem is, however, highly complex and is unlikely to be solved until the specificity of Z-enzyme is finally elucidated [cf. Peat, Thomas & Whelan (1952), Hopkins & Bird (1953) and Neufeld & Hassid (1955)].

SUMMARY

1. Starch has been isolated from the shoots and tubers of sprouting potatoes, and its physical and chemical properties have been compared with those of the starch from the original tubers.

2. Although the two tuber starches were similar, that from the shoot was profoundly different and possessed a lower iodine affinity and smaller granular size.

3. The amylose fractions separated from the different starches varied both with regard to molecular size and β-amylolysis limit, whereas the amylopectin fractions appeared to be similar.

4. The sedimentation of amylopectin has been studied.

5. The nature of the barrier to β-amylolysis is discussed.

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The Chemical Composition of Bull Semen with Special Reference to Nucleic Acids, Free Nucleotides and Free Amino Acids

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It has recently been shown that normal bull spermatozoa are able to incorporate amino acids into their proteins (Bhargava, 1957). As a preliminary to further work we have examined the nucleic acid, free nucleotide and free amino acid content of bull spermatozoa and seminal plasma, together with the general chemical composition of these spermatozoa. The nucleic acids of bull spermatozoa have received considerable attention in the past (Walker & Yates, 1952; Leuchtenberger, Leuchtenberger, Vendrely & Vendrely, 1952; Vendrely & Vendrely, 1953; Mann, 1954; Leuchtenberger, Murmanis, Murmanis, Ito & Weir, 1956), but no previous work seems to have been done on the free nucleotide content of these cells and there is only one earlier investigation (Gassner & Hopwood, 1952, 1953) on the free amino acids in bull semen. Other aspects of the chemistry of spermatozoa and seminal fluid have been extensively reviewed by Mann (1954).

Our results show that bull spermatozoa contain no ribonucleic acid, a large free nucleotide pool and a very small free amino acid pool. Bull seminal plasma was found to contain a higher concentration of free amino acids, and very low concentrations, if any, of free nucleotides and nucleic acids. This work has been carried out mainly on semen from only two bulls; this limitation was imposed partly by the necessity of using freshly collected semen from animals housed near a laboratory. Since the animals were of normal fertility, there is no reason to suppose that the results are not valid for such bulls generally.

MATERIALS AND METHODS

Semen. Bull semen was collected by the artificial-vagina technique (Walton, 1945) with precautions to avoid contamination of the sample with talc from the rubberware. The semen was collected, on the average, once a week. Each of the several ejaculates was examined separately under the microscope, and only those were mixed and used which appeared uncontaminated and did not contain an excessive number of dead spermatozoa.

The four bulls used are referred to as N_1, N_2, N_3 and N_4. Bull N_1 was a 3-years-old Friesian maintained at the National Institute for Medical Research. He had earlier been used for artificial-insemination purposes and was then shown to be of high fertility. Bulls N_2, N_3 and N_4 were in use at an artificial insemination centre (Cambridge) and were all of high fertility. The semen of bulls N_1, N_2, N_3 and N_4 is judged to be normal in all respects and it is for this reason that the prefix N has been used.

The majority of the observations recorded below on the chemical composition of semen were made on material from bulls N_1 and N_2. Unless otherwise stated, semen from bull N_1 was used within 2 hr. of collection. Semen from the remaining bulls was used about 3–4 hr. after collection. In all cases semen was kept at room temperature during the period between collection and use.

Concentration of spermatozoa. The concentration of spermatozoa in whole semen, or in suspensions of washed cells, was determined with a Fuchs–Rosenthal cytometer and a phase-contrast microscope, by the method of Bishop, Campbell, Hancock & Walton (1954). In experiments where analyses were made on washed spermatozoa, cell counts were also made on the rejected washes (these washes contained up to 10% of the cells originally present in the semen).

Buffers. The diluent hereafter referred to as bicarbonate buffer is Krebs–Ringer bicarbonate (Umbreit, Burris & Stauffer, 1957). The diluent referred to as fructose–bicarbonate buffer is Krebs–Ringer bicarbonate containing 5% (w/v) of fructose.

Dry weight of spermatozoa. Semen (2 ml.) was diluted with 4 ml. of fructose–bicarbonate buffer in weighed 15 ml. centrifuge tubes and centrifuged for 25 min. at 2500 rev./min. in an MSE Major centrifuge. The spermatozoa were washed once with 5 ml. of fructose–bicarbonate buffer and twice with 5 ml. of water. The cells were then dried to constant weight at 105° (4–5 hr.) in air.

General chemical composition of spermatozoa. Semen (4 ml.) was diluted and the cells were washed with buffer as described above. The cells were then suspended in 4·8 ml. of bicarbonate buffer and cooled to 0°. Ice-cold trichloroacetic acid [30% (w/v); 2·5 ml.] was added and the precipitate was washed once with 5 ml. of 5% (w/v) trichloroacetic acid, twice with 5 ml. lots of 5% NaCl and then twice with water (10 ml. lots). The residue was dried to constant weight *in vacuo* over $CaCl_2$ at room temperature. Subtraction of this weight from the dry weight of the cells gave the weight of the acid-soluble material. The residue was dissolved in 5 ml. of N-NaOH; the solution was rid of the small amount of insoluble residue by centrifuging and then mixed with 2·5 ml. of 60% (w/v) trichloroacetic acid. The precipitate was washed once with 5 ml. of 5% (w/v) trichloroacetic and then thrice with 10 ml. lots of water. The

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lipids were removed from the precipitate by successive washings (10 ml. of solvent each time), with ethanol (twice), acetone (twice), ethanol–ether (3:1, v/v; heating at 60° for 10 min.; twice) and finally once with ether. The lipid-free residue, consisting of proteins and nucleic acids, was then dried to constant weight. Nucleic acids were removed by heating the lipid-free precipitate with 8 ml. of 5% (w/v) trichloroacetic acid at 90° for 20 min. The residue was washed twice with 5% trichloroacetic acid, and once each with ethanol and with ether (10 ml. each). It was then dried at 105° to constant weight. Usually, 12–13% loss of weight occurred when proteins dried over $CaCl_2$ at room temperature were subjected to further drying at 105°. All values have been adjusted to dry weight at 105°.

Free nucleotides. Semen (1 ml.) was diluted with 5 ml. of fructose–bicarbonate buffer. The cells were separated by centrifuging, resuspended in fresh buffer (5 ml.) and centrifuged again. After wiping the inside of the tube, the cells were cooled to 0° and mixed with 5 ml. of ice-cold 0·5 M-$HClO_4$. In some experiments, a measured volume of the fructose–bicarbonate buffer washings, containing the diluted seminal plasma, was treated with an equal volume of M-$HClO_4$ at 0°. The precipitate in each case was removed as soon as possible by centrifuging at 0° (10 min. at 1000 *g*. for washed cells, and 20 min. at 20 000 *g* for seminal plasma) and the ultraviolet spectrum of the clear supernatant, containing the nucleotides, was determined with a Unicam spectrophotometer. Adjustment to pH 7·4 with NaOH did not significantly affect the spectrum obtained or the absorption at 260 mμ. With the extract from washed cells, the form of the absorption curve indicated that nucleotides were present in significant amounts and the nucleotide content was calculated assuming a molar-extinction coefficient of $E^{260}_{1\,cm.}$ (*P*) 10 000 (average mol.wt. taken to be 320, cf. Beaven, Holiday & Johnson, 1955). Duplicate runs on the same batch of semen gave identical results.

In a series of experiments with semen from bull N_1, the nucleotide estimations were made after storing the undiluted semen at room temperature for periods of 1, 3 and 4½ hr. After the appropriate time interval the semen was diluted with fructose–bicarbonate buffer, and centrifuged and the cells were washed as described above. The interval between first addition of buffer and addition of $HClO_4$ was 1 hr.

Nucleic acids. Nucleic acids were estimated in whole semen, in washed cells and in seminal plasma, from bulls N_1 and N_2.

Before treatment with trichloroacetic acid, the different samples were prepared as follows. Whole semen (1 ml.) was diluted with 3 ml. of fructose–bicarbonate buffer. Washed cells (from 1–5 ml. of semen) were suspended in 4 ml. of fructose–bicarbonate buffer. The seminal plasma examined was that material left in the supernatant after centrifuging samples of semen (1–5 ml.) previously diluted with an equal volume of fructose–bicarbonate buffer. Each sample was treated at 0° with half its volume of 30% (w/v) trichloroacetic acid, centrifuged, and the residue washed once with 5% (w/v) trichloroacetic acid and then twice with 5% NaCl at 0°. Lipids were removed as already described and the protein–nucleic acid residue was suspended in 5 ml. of M-$HClO_4$ at 4° for 18 hr. (cf. Ogur & Rosen, 1950). The mixture was then centrifuged at 0°. Ribose was estimated in 3 ml. of the supernatant by the method of Mejbaum (1939); 1 ml. was brought to pH 7·4 with NaOH, made up to 4 ml. with water and the ultraviolet spectrum was determined against a similarly treated $HClO_4$ blank. The ribonucleic acid (RNA) content was calculated assuming a statistical equimolarity in the tetranucleotide composition of RNA.

For estimation of deoxyribonucleic acid (DNA), the residue left after cold $HClO_4$ digestion was washed with two 5 ml. lots of M-$HClO_4$ at 0° and then extracted at 90° with 5 ml. and 3 ml. of M-$HClO_4$ (20 min. extractions). The combined hot $HClO_4$ extracts were brought to pH 7·4 with NaOH, made up to 10 ml. and the ultraviolet spectrum was determined. The DNA content was calculated from the absorption at 260 mμ, assuming that DNA has $E^{260}_{1\,cm.}$ (*P*) 8780 (Ogur & Rosen, 1950).

Free amino acids. Semen (2 ml.) was diluted with an equal volume of fructose–bicarbonate buffer and centrifuged. The supernatant was removed and the cells were resuspended in fructose–bicarbonate buffer and recentrifuged. The free amino acids were estimated in the residue (spermatozoa) by paper chromatography and in the combined supernatants (seminal plasma) by column chromatography, as described below.

Cells. The washed spermatozoa were suspended in water (2 ml.) and 30% (w/v) trichloroacetic acid was added (1·2 ml.). After 1 hr. at room temperature the precipitate was collected and washed twice with 2 ml. portions of 5% (w/v) trichloroacetic acid, the washings being added to the first extract. The extract was freed of trichloroacetic acid by repeated extraction with ether. The aqueous phase was added to a 3 cm. × 0·9 cm. column of Zeo-Karb 225 (150–200 mesh; hydrogen form) and the column was washed with about 20 ml. of water; during washing, no ninhydrin-positive material was eluted. The amino acids were then displaced with aq. 0·5 N-NH_3 soln., taken to dryness over H_2SO_4 and estimated by paper chromatography. The amino acid mixture was chromatographed on Whatman no. 1 paper, with phenol as solvent, in an atmosphere of NH_3. Serial dilutions of a standard mixture of amino acids were chromatographed on the same sheet of paper and the amount of each amino acid in the cell extract was estimated by visual matching with a corresponding spot from the standard mixture. Estimations made in this way are subject to a fairly large error (20%) but, in view of the small quantity of amino acids present in the cell extract, this method was considered the most appropriate.

Seminal plasma. The combined supernatants from the washed cells (diluted seminal plasma) were treated with 10% (w/v) trichloroacetic acid to give a final acid concentration of 5%. The residue was centrifuged, washed with trichloroacetic acid and the trichloroacetic acid removed from the combined washings with ether. Amino acids were isolated from the aqueous phase with Zeo-Karb 225 as described above. This material, from 2 ml. of semen, was then fractionated on a column of Zeo-Karb 225 WR 1·55, as described by Campbell, Jacobs, Work & Kressman (1955), and the individual amino acids were estimated with ninhydrin (Moore & Stein, 1948).

Breakdown of intracellular proteins. Spermatozoa were isolated from semen (4·5 ml.; bulls N_2 and N_4), after dilution with an equal volume of bicarbonate buffer, by centrifuging; they were then washed twice with buffer and finally suspended in a volume of buffer about equal to the volume of the original semen sample. Samples (1 ml.) of each suspension were incubated aerobically at 37° in 15 ml. centrifuge

tubes, one for each time point. At appropriate intervals (0, 30, 60, 120 and 240 min.), 0·3 ml. of 30% trichloroacetic acid was added. The precipitate was removed by centrifuging and washed with 1 ml. of 5% (w/v) trichloroacetic acid. Trichloroacetic acid was removed from the combined supernatants as already described, and the resulting aqueous phase was taken to dryness *in vacuo* over H_2SO_4 and made up to 1 ml. with water. Half (0·5 ml.) of this solution was used for direct estimation of total free amino nitrogen, with ninhydrin. On 0·3 ml. of the remainder, ninhydrin estimations were done after hydrolysis with 6N-HCl at 105° for 18 hr.

RESULTS AND DISCUSSION

General chemical composition of bull spermatozoa

The composition of bull spermatozoa, as found by us with the procedures described in the text, is given in Table 1. The dry weight recorded here ($16{\cdot}5 \times 10^{-9}$ mg./cell) is somewhat lower than that reported earlier (Zittle & O'Dell, 1941), but this may be due to differences in the drying technique. Our value is for cells dried at 105°. One observation requires special comment, namely the large loss of weight produced by treating the trichloroacetic acid-precipitable material with organic solvents. This procedure would normally be expected to extract only lipid and phospholipid, but, in the present work, appreciable quantities of protein were also extracted by the organic solvents. These proteins became progressively more insoluble on repeated dissolution in, and evaporation from, alcoholic solutions. In later experiments on protein metabolism in spermatozoa (Bhargava, Bishop & Work, 1959), extraction with organic solvent was carried out after removal of DNA with hot trichloroacetic acid. Even under these conditions, some protein was extracted by organic solvents. It seems likely that this is due to the presence of much protamine-like material and phospholipid which form complexes soluble in organic solvents (cf. Chargaff, 1938).

Table 1. *Composition of normal bull spermatozoa*

Estimations were made on cells derived from 3 ml. of semen, as described in the text. The results quoted are mean values of three determinations on bull N_1.

	$10^9 \times$ Amount per cell (mg.)	Percentage of dry weight
Dry weight	16·5	100
Trichloroacetic acid-soluble material	3·3	20
Alkali-insoluble material	0·17	1·3
Lipid and lipoprotein*	6·4	39
Nucleic acid	3·4	21
Protein other than lipoprotein	3·2	19

* The term 'lipoprotein' is used for protein extractable into organic solvent under conditions defined in the text.

Deoxyribonucleic acid of bull spermatozoa and seminal plasma

The DNA content of bull spermatozoa was found to be $3{\cdot}4 \times 10^{-9}$ mg./cell (Table 2). This figure agrees with that reported by Vendrely & Vendrely (1953), but values as low as $2{\cdot}8 \times 10^{-9}$ mg./cell (Mirsky & Ris, 1949) and as high as $4{\cdot}5 \times 10^{-9}$ mg./cell (Zittle & O'Dell, 1941) have been reported. In the present work, the same DNA value per cell was obtained when either whole semen or washed cells were used.

Ribonucleic acid of bull spermatozoa and seminal plasma

A small amount of ribose-containing material was detected in spermatozoa from bull N_1 and a similar amount in cells from bull N_2 (Table 2). Calculated as a percentage of the DNA present it is less than 1%. The RNA content of nuclei from other cells is invariably much higher, though it varies over wide limits among cell types, and probably lies between 10 and 30% of the DNA content for most cells (Dounce, 1955). It is likely that the true figure for bull spermatozoa is substantially below 1%, since, owing to the enormous excess of DNA, estimations of ribose will be somewhat high owing to interference by deoxyribose.

The seminal plasma of both bulls contained small and variable amounts of ribose-containing material. This may be RNA derived from cytoplasmic droplets (Bishop & Austin, 1957) and its presence accounts for the slightly higher RNA values obtained when whole semen is used instead of washed cells (Table 2).

The ultraviolet spectrum of the cold perchloric acid digests derived from washed spermatozoa

Table 2. *Nucleic acid content of bull spermatozoa*

All DNA and RNA estimations were on material from the same batch of semen.

Bull	Fraction	Vol. of semen used (ml.)	No. of cells used (millions)	DNA (mg./10^9 cells)	RNA† (mg./10^9 cells)	RNA (as percentage of DNA)
N_1	Semen	1·0	2350	3·30	0·050	1·5
	Cells	1·8*	6680	3·34	0·025	0·75
N_2	Cells	2·0*	1880	3·43	0·007	0·2

* Amount of semen from which the cells were derived.

† Based on ribose estimations.

resembles that of nucleic acid (Ogur & Rosen, 1950). Comparison of the RNA values calculated from extinction at 260 mμ with those obtained by the ribose method indicates that, in addition to the small amount of RNA, some DNA also had been extracted. This DNA, however, represented only a small fraction (less than 5%) of the DNA extracted by hot perchloric acid. The RNA content as estimated by the ribose method was about $0{\cdot}015 \times 10^{-9}$ mg./cell, but the apparent RNA value as determined by ultraviolet absorption on the perchloric acid extract was $0{\cdot}26 \times 10^{-9}$ mg./cell. Our results show therefore that spectrophotometric estimation of RNA by the method of Ogur & Rosen (1950) is likely to give erroneous results when the RNA content of the material under examination is low.

Table 3. *Free nucleotide content of spermatozoa*

Experiments bearing the same numbers were done on a single batch of semen.

Expt. no.	Bull	No. of cells used (millions)	Amount of nucleotide (mg./10^9 cells)	Period of storage of semen after collection (min.)
1	N_1	1600	0·184	120
2	N_1	1440	0·158	150
3	N_1	1130	0·090	60
3	N_1	1130	0·071	180
3	N_1	1130	0·078	270
4	N_2	1580	0·120	120

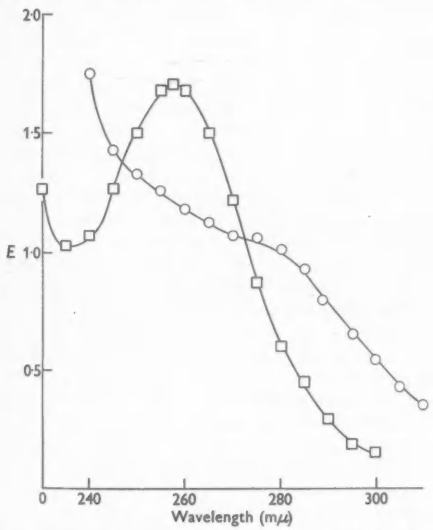

Fig. 1. Ultraviolet spectra of the acid-soluble fractions derived from the spermatozoa (□) and from the seminal plasma (○) of bull N_1.

The present conclusion that bull spermatozoa contain, at most, only traces of RNA is in agreement with other work in which different kinds of spermatozoa have been examined by a variety of methods. Mauritzen, Roy & Stedman (1952) could not detect any RNA in fish sperm heads; White, Leslie & Davidson (1953) found no RNA in human spermatozoa and Mann (1954) found none in ram spermatozoa. The value for RNA in bull spermatozoa reported by Vendrely & Vendrely (1948) is probably attributable to contamination with non-RNA phosphorus, as is suggested by the later work of Mauritzen *et al.* (1952).

Intracellular free nucleotides

When washed spermatozoa from bull N_1 or N_2 were extracted with ice-cold perchloric acid, an extract was obtained which had the typical absorption spectrum of nucleotides (Fig. 1). It is unusual to obtain by direct extraction of cells with cold perchloric acid an extract which is clearly mainly nucleotide. We have found, however, that spermatozoa have a particularly low free amino acid pool together with an exceptionally large free nucleotide pool. The nucleotide values obtained are given in Table 3. Since the free nucleotides were not purified, these values must be regarded as approximations. The size of the nucleotide pool varied somewhat with different batches of semen (range 0·4–1·1% of dry weight). No appreciable loss of nucleotide from the cells was observed during storage of semen for periods of up to 4½ hr. at room temperature. The ultraviolet-absorption spectrum (Fig. 1) of the acid-soluble fraction derived from seminal plasma indicated that free nucleotides are present, at most, in traces.

Free amino acids of seminal plasma and spermatozoa

The values for free amino acids found in the seminal plasma of bull N_1 are given in Table 4. Since the estimations were made by the method of Moore & Stein (1951) after chromatographic separation on ion-exchange resin, the values for the major components can be regarded as fairly accurate (±5%). In addition, a number of minor components with ninhydrin-positive reactions were detected. Some of these may have been peptides but no attempt was made to identify them. The free amino acid content of the washed cells was too low to permit accurate estimation, but approximate values were obtained by paper chromatography (Table 4).

Gassner & Hopwood (1952, 1953), using quantitative paper chromatography, determined the free amino acids in whole semen and in seminal plasma. They found no difference between whole semen and seminal plasma, which suggested, in agreement

with our results, that bull spermatozoa have a small amino acid pool. Their values for whole semen are considerably lower than our values for seminal plasma, and they were able to find only five amino acids (glutamic acid, aspartic acid, glycine, serine and alanine). A possible source of this discrepancy is that the column method detected components missed in paper chromatograms. It is also possible that Gassner & Hopwood analysed their specimens sooner after ejaculation than we have done. A fairly rapid increase in amino nitrogen on storage of semen has been reported by Lundquist (1949, 1952) and by Jacobsson (1950). Since we were primarily interested in the size of the amino acid pool during periods of incubation of spermatozoa with isotopically labelled amino acids (Bhargava *et al.* 1959), it seemed preferable to estimate the amino acid pool at about 1 hr. after ejaculation, the time at which incubations were usually set up.

Our values for the intracellular amino acid pool of bull spermatozoa (1·07 m-moles/100 g. dry wt.) are low compared with those reported for other mammalian tissues, which seem to exceed 5 m-moles/100 g. dry weight (cf. Tallan, 1955; Smith & Nelson, 1957).

Table 4. *Free amino acid pool of spermatozoa and seminal plasma from bull* N_1

Where no amino acid was detected this is indicated as ND. The concentration of spermatozoa in the semen used was 1525×10^6/ml.

Amino acid	Amino acid (μmole/ml. of semen) Cells	Seminal plasma
Aspartic acid	ND	0·32
Threonine	ND	0·15
Serine	0·04	0·62
Glutamic acid	0·08	2·79
Glycine	0·07	0·85
Alanine	0·04	0·49
Valine	0·02	0·15
Leucine	0·02	ND
Isoleucine	ND	ND
Tyrosine	ND	0·13
Lysine	ND	0·11
Histidine	ND	Trace
Arginine	ND	0·11
Total	0·27	5·72

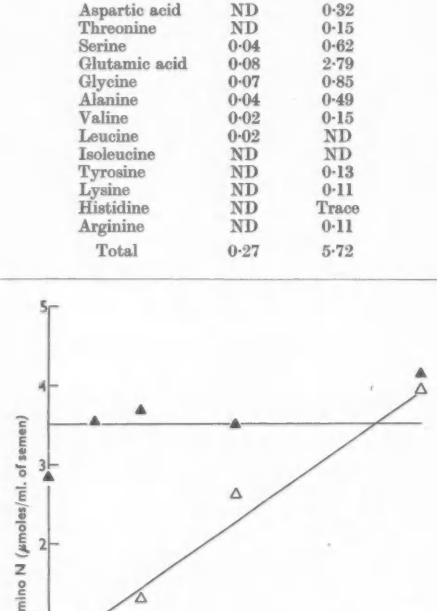

Fig. 2. Release of amino nitrogen (△) and peptides (▲) during incubation of spermatozoa in bicarbonate buffer. (For conditions of incubation see text.)

Proteolysis during incubation

In earlier experiments (Bhargava, 1957) it was noted that there was some loss of protein during incubation of spermatozoa at 37°. This observation prompted us to determine the rate of release of amino acids and peptides during incubation of washed spermatozoa in bicarbonate buffer. Free amino nitrogen was determined directly with ninhydrin, and trichloroacetic acid-soluble peptides were estimated with the same reagent after hydrolysis. The results, given in Fig. 2, show that there was a linear increase in the concentration of amino nitrogen during incubation. This increased amino nitrogen must be derived ultimately from cell peptides or cell proteins.

In these experiments, the values for free amino nitrogen at zero incubation time are higher than those in the experiment reported in Table 4, but this discrepancy is not unexpected since the values in Table 4 exclude ammonia, whereas ammonia would be estimated along with amino acids in the incubation experiments. Although there was a substantial increase in total amino nitrogen on incubation, there was no increase in total peptide, and subtraction of the free amino nitrogen values from the total amino nitrogen values results in a straight line parallel to the base line (Fig. 2). We conclude therefore that any peptides released during incubation undergo further proteolysis to free amino acids.

SUMMARY

1. Data are presented on the chemical composition of bull spermatozoa and seminal plasma.

2. The dry weight of the spermatozoon was found to be $16{\cdot}5 \times 10^{-9}$ mg., of which $3{\cdot}38 \times 10^{-9}$ mg. was deoxyribonucleic acid. Ribonucleic acid, if present at all, occurred only in trace amounts.

3. The free nucleotide content of bull spermatozoa is unexpectedly large and constitutes on average 0·75 % of the dry weight of the cell.

4. The spermatozoa contain only traces of a few free amino acids. The seminal plasma contains 5·72 μmoles of total amino acids/ml.

5. The spermatozoon proteins were found to undergo slow degradation to free amino acids on incubation at 37°.

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Incorporation of [^{14}C]Amino Acids into the Proteins of Bull Spermatozoa

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During the last two decades, a considerable amount of evidence has accumulated to suggest that ribonucleic acid plays an essential role in protein biosynthesis (see Brachet, 1955). Since bull spermatozoa are virtually devoid of ribonucleic acid (Bhargava, Bishop & Work, 1959), they would be expected to be incapable of protein synthesis. It has already been shown, however, that washed bull spermatozoa can incorporate radioactive amino acids into their proteins (Bhargava, 1957); these observations are extended and evaluated in the present paper. We have also studied amino acid incorporation by unwashed spermatozoa in seminal plasma since this system more closely resembles that met with *in vivo*. Marked differences were observed between the washed and the unwashed cells in both the rates and the patterns of incorporation of amino acid into protein. The effect of several metabolic inhibitors on incorporation by both washed and unwashed cells was also investigated. For comparative purposes, the rate of incorporation of amino acids into proteins of reticulocytes has been measured under similar conditions to those used for washed spermatozoa.

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EXPERIMENTAL

Materials and methods

Semen. The bull spermatozoa used were obtained from five normal fertile bulls. These bulls (N_1, N_2, N_3, N_4 and N_5) together with the method of collecting the semen and the method of determining the concentration of spermatozoa, have been described in the preceding paper (Bhargava *et al.* 1959). Bull N_1 was the animal used in an earlier investigation (Bhargava, 1957).

Radioactive amino acids. Dried *Chlorella protein* generally labelled with ^{14}C (4·8 mg., specific activity 102 μc/mg.; the Radiochemical Centre, Amersham, Bucks.), was hydrolysed with 2 ml. of 6 N-HCl. The hydrolysate was evaporated to dryness, extracted with water and the extract made up to 10·6 ml.; it was then stirred with 0·5 g. of Celite (grade 519 A, Johns-Manville Co. Ltd.) to remove suspended material and centrifuged. The supernatant, subsequently referred to as [^{14}C]*Chlorella* hydrolysate, contained about 4·5 μmoles of mixed amino acids and 46 μc of radioactive carbon/ml. In a few experiments, *Chlorella* protein of half this specific activity was used; in these experiments the observed counts have been doubled so as to facilitate comparison between experiments.

Radioactive valine (specific activity 8 μc/μmole; The Radiochemical Centre, Amersham, Bucks.) was dissolved in water to a concentration of 4·5 μmoles/ml. All data obtained with [^{14}C]*valine* have been adjusted to correspond to a value of 10·2 μc/μmole (the same specific activity as the standard *Chlorella* hydrolysate). In this way, direct comparison can be made between results with [^{14}C]*Chlorella* hydrolysate and those with [^{14}C]valine.

Metabolic inhibitors. The following inhibitors were used: chloramphenicol, 2:4-dinitrophenol, sodium fluoride, iodoacetic acid (neutralized to pH 7·0), 8-azaguanine, *p*-fluorophenylalanine and ribonuclease. Solutions to be tested were prepared in water (dinitrophenol was in 1 % $NaHCO_3$ soln.) and the appropriate amount was evaporated to dryness in the tubes in which the cells were to be incubated. *p*-Fluorophenylalanine was synthesized in this Laboratory; ribonuclease was prepared by Dr R. R. Porter; all other inhibitors were commercial preparations.

Spermatozoon motility. Estimates of spermatozoon motility were made on the fresh undiluted semen, on the washed cell suspensions just before starting incubation and on samples taken out after incubation for various periods of time. The ratings were made by the method of Bishop & Salisbury (1955*a*). All samples were rated by the same person.

Bacterial counts. In some experiments estimations were made of the number of bacteria present before incubation and after incubation of semen or washed spermatozoa for various lengths of time. Bacterial counts were determined, after suitable dilution, by plating on blood–agar plates and incubating aerobically at 34° for 20 hr.

Preparation of cell suspensions

Suspensions of washed spermatozoa. Portions of semen (0·6–1·4 ml.) were pipetted into 15 ml. round-bottomed centrifuge tubes, diluted with 3–5 vol. of Krebs–Ringer bicarbonate buffer, pH 7·4 (Umbreit, Burris & Stauffer, 1957; referred to below as bicarbonate buffer), or Krebs–Ringer bicarbonate buffer containing 5 % (w/v) of fructose (subsequently called fructose–bicarbonate buffer), and centrifuged for 5–20 min. at 2500 rev./min. (MSE Major centrifuge). Different batches of semen differed in the rate and extent of sedimentation of the spermatozoa. The cell sediment was resuspended in fructose–bicarbonate buffer (3–9 ml.). When the effect of omitting fructose was studied, the cells were washed with and resuspended in bicarbonate buffer. The concentration of spermatozoa was determined in the combined supernatants; in some experiments the first supernatant, consisting of diluted seminal plasma, was also used for incubation. In those experiments in which the washed cells had to be divided into a large number of samples, the cells were washed in one lot, resuspended in buffer and samples of the suspension were dispensed into the tubes used for incubation. In experiments in which the effect of varying cell concentration on the incorporation of [^{14}C]*Chlorella* hydrolysate was studied, 1, 2, 3 and 5 ml. volumes of the cell suspension were pipetted into separate tubes and each was made up to 5 ml. with fructose–bicarbonate buffer.

Suspensions of reticulocytes. A rabbit (3 kg.) was injected intravenously with 50 mg. of *N*-acetylphenylhydrazine in 5 ml. of 0·9 % NaCl soln. each day for 5 days (Cruz, 1941). On the second day after the last injection, the reticulocyte content, as determined by the method of Cruz (1941), was 80–90 % of all blood cells. Next day the rabbit was bled into sterile 0·9 % NaCl soln. containing heparin. The cells were washed twice with 0·9 % NaCl, twice with sterile bicarbonate buffer and then suspended in a volume of bicarbonate buffer equal to the original volume of blood. After determination of the cell count, the suspension was further diluted with the buffer to give the desired cell concentrations.

Incubations

Whole semen, spermatozoa and seminal plasma. Samples of semen, washed cell suspension or diluted seminal plasma (for quantities see individual Tables) were dispensed into 15 ml. round-bottomed centrifuge tubes and [^{14}C]*Chlorella* hydrolysate or [^{14}C]valine solution was added. The tubes were incubated at 37° with shaking (100 cyc./min.). Unless otherwise stated, all incubations were carried out in air or O_2+CO_2 (95:5), the results being substantially the same in either case. At the appropriate time, samples were taken out for motility determinations and for bacterial counts, and to the remainder a sufficient volume of 30 % (w/v) trichloroacetic acid was added to give a final concentration of 10 %.

To check that incorporation was taking place solely in intracellular proteins, some semen samples (1·2 ml.) were diluted, after incubation, with 7·5 ml. of fructose–bicarbonate buffer. The diluted samples were centrifuged for 30 min. (regarded as part of the incubation period) and the supernatant was decanted and mixed with 4 ml. of 30 % (w/v) trichloroacetic acid; the sedimented cells were suspended in 3 ml. of fructose–bicarbonate buffer and precipitated with 2 ml. of 30 % trichloroacetic acid.

In other experiments, the effect of replacing seminal plasma with an equal volume of fructose–bicarbonate buffer was studied. The semen (0·75 ml.) was diluted with 5 ml. of fructose–bicarbonate buffer, centrifuged and the supernatant decanted. The inside of the tube was wiped with filter paper and the cells were resuspended in 0·55 ml. of fresh buffer together with 0·15 ml. of [^{14}C]*Chlorella*

hydrolysate and incubated at 37°. Proteins were precipitated by the method described for semen.

Reticulocytes. For experiments on the incorporation of [^{14}C]*Chlorella* hydrolysate, 2 and 8 ml. samples of the reticulocyte suspension were diluted to 24 ml. with bicarbonate buffer and each was incubated at 37° with 0·24 ml. of [^{14}C]*Chlorella* hydrolysate in an atmosphere of O_2+CO_2 (95:5). At appropriate times, 5 ml. samples were taken out of each flask and mixed with 2 ml. of 30% (w/v) trichloroacetic acid. Finally, a 2 ml. sample was taken out for determination of protein content.

For the experiment on the incorporation of [^{14}C]valine, three samples (2 ml.) of the reticulocyte suspension were diluted to 18 ml. with bicarbonate buffer in three flasks, and 2 mg. and 20 mg. of non-radioactive valine was added to flasks 2 and 3, followed by 0·29 ml. of the [^{14}C]valine solution in all flasks. The mixture was incubated at 37° under O_2+CO_2 (95:5) and, at appropriate time intervals, 4 ml. samples were removed from each flask and mixed with trichloroacetic acid.

In all experiments, samples for the zero-time point were precipitated with trichloroacetic acid immediately after addition of the radioactive amino acid.

Isolation of proteins

Semen, spermatozoa and seminal plasma. After precipitation with 30% (w/v) trichloroacetic acid, the mixture was left to stand for at least 15 min. It was then centrifuged, washed once with 5% trichloroacetic acid and once with 5% (w/v) NaCl. (With spermatozoa, the supernatants were invariably clear, but with seminal plasma or whole semen protein, the washes were often turbid. The finely suspended material in these washes could be removed by prolonged centrifuging at 5000 rev./min., but in general this was not done.) The residue obtained after the NaCl wash was dissolved in N-NaOH (1–3 ml., depending on the volume of semen used). The seminal plasma proteins went into solution easily at room temperature. The spermatozoon proteins dissolved in alkali solution only on heating at 70° for 1–2 min. Non-radioactive bovine serum-albumin hydrolysate (0·2 ml., corresponding to approx. 4 mg. of mixed amino acids) or valine (4 mg. in 0·2 ml.), as appropriate, was added and the mixture centrifuged to remove alkali-insoluble material, normally present in trace amounts. The alkali solution was treated with an equal volume of 30% (w/v) trichloroacetic acid. The precipitate was washed once with 5% trichloroacetic acid, then heated with 5% trichloroacetic acid at 90° for 20 min., washed once again with 5% trichloroacetic acid at room temperature and, to remove lipids, was washed twice with ethanol, twice with acetone, twice with ethanol–ether (3:1, v/v) and finally once with ether. All washings were done with 4–5 ml. of the wash fluid.

Reticulocytes. The precipitate obtained on addition of trichloroacetic acid to the incubation mixture was washed once with 5% (w/v) trichloroacetic acid and then with 5 ml. of acetone containing a few drops of 30% trichloroacetic acid at the boiling point of the mixture (to remove porphyrin). The residue was heated with 5% trichloroacetic at 80° for 20 min. and then dissolved in N-NaOH containing an excess of serum-albumin hydrolysate. The alkaline solution was mixed with an equal volume of 30% (w/v) trichloroacetic acid and the precipitate was washed twice with 5% trichloroacetic acid, once with ethanol and once with ethanol–ether (3:1, v/v). The entire process from dissolution in alkali onwards was then repeated, and the proteins obtained were finally washed with ether.

Measurement of radioactivity

Protein was plated as a slurry in 30% (v/v) ethanol (ether for reticulocyte protein) on preweighed 2 cm.^{2} aluminium planchets. After drying over $CaCl_2$ the plates were reweighed. Radioactivity was determined in a thin-window Geiger–Müller counter. A standard Perspex disk of infinite thickness, 1 cm.^{2} area and specific activity 10^{-3} μc/mg., gave 1000 counts/min. on this counter. Corrections for self-absorption were made with the data given for wax by Calvin, Heidelberger, Reid, Tolbert & Yankwich (1949). Specific activities are expressed as counts/min./mg. of protein. A small variable amount of radioactivity was always found associated with proteins obtained at zero time (i.e. after addition of radioactive amino acids followed by immediate precipitation). Unless otherwise stated, specific activity at zero time has been subtracted from the values obtained at other times.

With [^{14}C]valine, a specific activity of 1000 counts/mg. of protein corresponds to an incorporation of 0·275 μmole of radioactive amino acid/g. of protein.

Treatment of proteins with performic acid. Performic acid (1 ml.; prepared by the method of Hirs, 1956) was added to labelled spermatozoon protein in solution in formic acid at 0°. The mixture was held at 0° for 2 hr. and then mixed with 0·5 ml. of either casein hydrolysate solution or valine solution (both containing about 10 mg. of amino acid) as appropriate. It was then dried *in vacuo* at 0° over P_2O_5; the residue was dissolved in N-NaOH, precipitated with trichloroacetic acid and the precipitate was washed and dried in the usual way.

RESULTS

Incorporation of mixed [^{14}C]*amino acids into bull spermatozoa*

The incorporation of [^{14}C]*Chlorella* hydrolysate into the proteins of washed spermatozoa was usually linear for about 2 hr. The results of a typical experiment are given in Fig. 1. Considerable variations in incorporation rate were observed from experiment to experiment, and the results suggest (Table 1) that there may have been a seasonal change in rate. By contrast, the incorporation of amino acids from [^{14}C]*Chlorella* hydrolysate into whole semen proceeded linearly for only 20 min. and then ceased. Fig. 2 gives the results of a typical experiment with bull N_1. Variations between experiments with bull N_1 over a period of 6 months are given in Table 2, along with results of experiments with semen from four other bulls (N_2, N_3, N_4 and N_5). As can be seen from Table 2, there was only one exceptional experiment, during which incorporation continued for 2 hr.

There was no incorporation in diluted seminal plasma (Fig. 1). Since seminal plasma freed from spermatozoa did not incorporate radioactive amino acids, it seemed likely that incorporation of amino

Table 1. *Variation in the rate of incorporation of* [^{14}C]Chlorella *hydrolysate into the proteins of washed bull spermatozoa*

For details of incubation and the method of isolating proteins see Fig. 1 and text. In all experiments except no. 28 the initial motility was good, and was maintained nearly unchanged for 3 hr. In Expt. 28 the initial motility was poor, and was nil at 3 hr.

Expt. no.	Date	Cell concentration (millions/ml.)	Concn. of [^{14}C]amino acid (mM)	Specific activity of proteins (counts/min./mg.) 1 hr.	1½ hr.	3 hr.	3½ hr.	4½ hr.
1	20. xii. 56	Not counted	0·050	422	—	—	—	—
2	10. i. 57	210	0·050	254	—	583	—	708 (5 hr.)
28	9. v. 57	235	0·075	183	—	294	—	—
34	29. v. 57	155	0·075	—	173	240	—	—
44	3. vii. 57	268	0·075	—	70	—	153	162
59	7. viii. 57	188	0·075	—	52	89	—	—
60	7. viii. 57	188	0·075	—	52	—	129	142
61	23. i. 58	Not counted	0·075	86	—	135	—	—
62	27. iii. 58	157	0·075	51	103 (2 hr.)	—	—	—

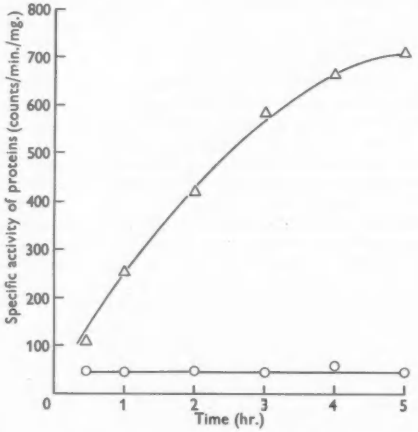

Fig. 1. Incorporation of [^{14}C]*Chlorella* hydrolysate into the proteins of washed spermatozoa (△) and diluted seminal plasma (○) from bull N_1. For each time point, 4·5 ml. of a suspension of washed cells (210×10^6 cells/ml.) or diluted seminal plasma was incubated with 0·05 ml. of the [^{14}C]-*Chlorella* hydrolysate. Proteins were isolated as described in the text. Specific activities have not been corrected for radioactivity at zero incubation time.

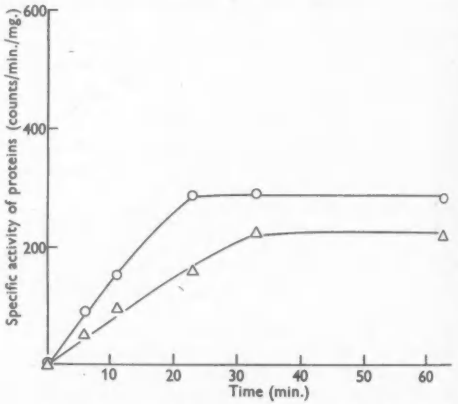

Fig. 2. Incorporation of [^{14}C]*Chlorella* hydrolysate into the proteins of bull semen (○, ethanol-insoluble proteins; △, ethanol-soluble proteins). Semen (1·2 ml. for each time point; 1220×10^6 cells/ml.) from bull N_1 was incubated with 0·3 ml. of [^{14}C]*Chlorella* hydrolysate and proteins were isolated as described in the text.

acids into whole semen was also exclusively intracellular. To confirm this, spermatozoa and seminal plasma were separated by centrifuging after various periods of incubation and the two kinds of protein examined separately. Radioactivity was found in proteins from spermatozoa whereas no appreciable uptake was observed in those from seminal plasma (Table 3).

In one experiment (Fig. 3) with semen from bull N_1, the seminal plasma was removed and the spermatozoa were incubated with [^{14}C]*Chlorella* hydrolysate in a volume of fructose buffer equal to that of the original semen. The results, which resemble those with washed cells, show that the differences in the pattern of incorporation observed between whole semen and washed spermatozoa are not caused by differences in cell concentration. It was found in other experiments that dilution of semen with buffer shifted the pattern of incorporation from that characteristic of whole semen towards that characteristic of the washed cells.

Incorporation of valine

Since studies on protein synthesis in other systems have usually been carried out with a single labelled amino acid, we have studied also the incorporation of valine. The incorporation of [^{14}C]-valine (Fig. 4) into the proteins of washed spermatozoa, was similar (except for an initial lag) to that

Table 2. *Variations between experiments in the incorporation of* [^{14}C]Chlorella *hydrolysate into the proteins of bull semen*

Semen (0·3–1·2 ml.) was incubated with 25% (in Expt. 11, 33%) of its own volume of [^{14}C]*Chlorella* hydrolysate. The proteins were isolated as described in the text. All experiments with bull N_1 were started within 1½ hr. of collection of the semen. The experiments with bulls N_2–N_5 were begun about 4 hr. after collection.

Expt. no.	Date	Bull	Cell concentration (millions/ml. of semen)	Specific activity of proteins (counts/min./mg.) 15 min.	30 min.	1 hr.	2 hr.	3 hr.	4 hr.
5	28. ii. 57	N_1	1220	284*	288	282	—	—	—
7	18. iii. 57	N_1	1100	—	46	48	—	—	—
11	29. iii. 57	N_1	Not counted	—	210	—	—	—	297
12	4. iv. 57	N_1	1280	—	171	—	168†	—	191
24	2. v. 57	N_1	1670	—	51	56	—	48‡	—
30	22. v. 57	N_1	2430	—	81	138	186	181	191
37	1. vi. 57	N_1	1340	—	—	51	—	57	—
42	1. vii. 57	N_2	760	45	49	53	—	—	—
42	1. vii. 57	N_3	570	58	58	56	—	—	—
42	1. vii. 57	N_4	1650	30	42	41	—	—	—
42	1. vii. 57	N_5	1470	29	32	—	—	—	—

* 20 min. † 1½ hr. ‡ 2½ hr.

Table 3. *Radioactivity in the proteins of spermatozoa and of seminal plasma after incubation of normal semen with* [^{14}C]Chlorella *hydrolysate*

Semen from bull N_1 (1·2 ml. for each time point, 1540×10^6 cells/ml.) was incubated with 0·3 ml. of [^{14}C]-*Chlorella* hydrolysate and the spermatozoa were separated by centrifuging. Proteins were isolated as described in the text.

Time of incubation (hr.)	Specific activity of proteins (counts/min./mg.) Spermatozoa	Seminal plasma*
0	0	0
½	600	0
1½	593	49
3½	618	70
5	520	70
6½	579	70
8	583	70

* It is difficult to obtain a plasma sample entirely free from spermatozoa and the small residual radioactivity found is due at least in part to contamination of the plasma with cells (cf. Fig. 1).

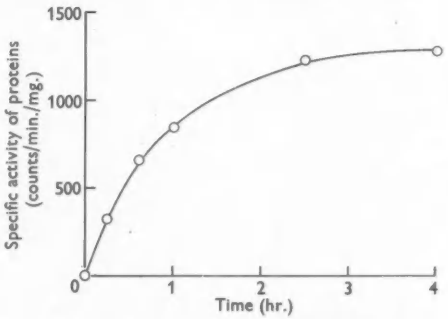

Fig. 3. Effect of replacing seminal plasma with an equal volume of fructose–bicarbonate buffer on the incorporation of [^{14}C]*Chlorella* hydrolysate into the proteins of bull spermatozoa. For each time point, cells from 0·6 ml. of semen (1840×10^6 cells/ml.) were suspended in 0·55 ml. of fructose–bicarbonate buffer and incubated with 0·15 ml. of [^{14}C]*Chlorella* hydrolysate. Proteins were isolated as described in the text.

obtained with [^{14}C]*Chlorella* hydrolysate. Variation in the rate of valine incorporation in three experiments is shown in Table 4. The incorporation of [^{14}C]valine into proteins of whole semen was almost negligible, as shown in Table 5.

Effect of storage of semen

During the course of this work, it occurred to us that some of the variation encountered from experiment to experiment in the extent of incorporation of radioactive amino acids into spermatozoa might be caused by differences in the time elapsing between the collection of the semen and the beginning of the experiment, or by differences in the concentration of the cells in the suspensions used. Table 6 shows fluctuations in the rate of amino acid incorporation after different periods of storage of semen at room temperature. Fig. 5 shows that the concentration of the cells in the incubation mixture is one factor influencing the rate of uptake of radioactive amino acids, the two parameters being inversely related.

Ethanol-soluble proteins

The recovery of protein per cell, after precipitation with trichloroacetic acid and removal of lipids and DNA, varied from experiment to experiment. These differences were caused by extraction of varying amounts of protein by organic solvents

Table 4. *Variation in the rate of incorporation of* [^{14}C]*valine into the proteins of washed spermatozoa*

For details of incubation and method of isolating proteins see text.

Expt. no.	Date	Cell concentration (millions/ml.)	Concn. of [^{14}C]valine (mM)	Specific activity of proteins (counts/min./mg.) ½ hr.	1 hr.	2 hr.	3 hr.	4 hr.
15	10. iv. 57	—	0·150	7	150	231	—	—
20	23. iv. 57	113	0·100	8	100	160	—	—
45	9. vii. 57	148	0·075	7	24	42	65	73

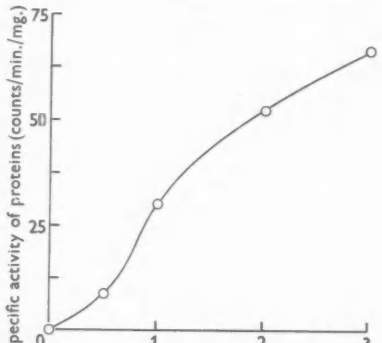

Fig. 4. Incorporation of [^{14}C]valine into the proteins of washed spermatozoa. For each time point, 3 ml. of the cell suspension (148×10^6 cells/ml.) and 0·05 ml. of [^{14}C]-valine solution was used. Proteins were isolated as described in the text.

Table 5. *Incorporation of* [^{14}C]*valine into the proteins of normal semen (bull* N_1)

In Expts. 14 and 46, 0·6 and 0·3 ml. of semen was used respectively. After incubation with [^{14}C]valine, the proteins were isolated as described in the text. In Expt. 14, 0·1 ml. of [^{14}C]valine solution was used; in Expt. 46, 0·075 ml. was used.

Time (hr.)	Specific activity of proteins (counts/min./mg.) Expt. 14	Expt. 46
0	0	0
½	2·7	6·7
1	3·9	—
1½	—	6·2
3	4·9	8·7

(mostly by ethanol) used for removing lipids (cf. Bhargava *et al.* 1959). The protein extracted in this way could be rendered insoluble in ethanol by repeated dissolution in ethanol and evaporation of the solvent at 80°. The protein had much the same specific activity as the ethanol-insoluble protein (Fig. 2). In whole semen the seminal plasma usually accounted for about half the total protein.

Table 6. *Effect of storage of whole semen on the incorporation of* [^{14}C]Chlorella *hydrolysate into proteins of washed normal spermatozoa*

Spermatozoa were stored for the indicated times in whole semen at room temperature, then washed and incubated for 2 hr. with [^{14}C]*Chlorella* hydrolysate (0·1 ml.) and the proteins isolated as described in the text. In Expt. 1, 1570×10^6 cells were suspended in 3·3 ml. of fructose-bicarbonate buffer for each time point; in Expt. 2, 3·6 ml. of buffer contained 1656×10^6 cells.

Storage time (min.)	Specific activity of proteins (counts/min./mg.) Expt. 1 Ethanol-insoluble proteins	Expt. 2 Ethanol-insoluble proteins	Expt. 2 Ethanol-soluble proteins*
10	—	180	—
30	156	—	—
75	219	—	—
90	—	129	142
120	153	—	—
180	—	304	283
210	142	—	—
360	93	—	—

* From the combined lipid washes (see text). This material was shown to be protein by nitrogen estimation and estimation of total amino acids after hydrolysis.

Incorporation of [^{14}C]*amino acids into reticulocytes*

Reticulocytes are known to have a high RNA content and (as measured by radioactive amino acid incorporation) a high rate of protein turnover (Borsook, Deasy, Haagen-Smit, Keighley & Lowy, 1952).

Our observations on uptake of [^{14}C]*Chlorella* hydrolysate and of [^{14}C]valine by rabbit reticulocytes under conditions similar to those used for washed spermatozoa are shown in Figs. 6 and 7. For reticulocytes suspended in buffer at a total protein concentration of 3 mg./ml. we obtained valine incorporation rates of 0·36, 0·43 and 0·47 μmole of amino acid/g. of protein/hr., with 0·095, 0·996 and 9·94 μmoles of valine/ml., respectively. This is in fair agreement with results obtained by the above-mentioned workers. With [^{14}C]*Chlorella* hydrolysate (0·045 μmole of labelled amino acids/ml. and with a reticulocyte suspension

containing 2·35 mg. of protein/ml.), an incorporation rate of 0·75 μmole of labelled amino acids/g. of protein/hr. was observed. Reticulocytes resembled washed spermatozoa in that a decrease in cell concentration was accompanied by an increased rate of incorporation (Fig. 6).

Effect of inhibitors

Since protein synthesis by spermatozoa in the absence of RNA was unexpected, the effects of known inhibitors of protein synthesis were studied, in an effort to evaluate the significance of the observed amino acid incorporation. As can be seen from Table 7, ribonuclease and azaguanine were without effect, whereas fluoride had a slight inhibitory effect on incorporation of mixed [^{14}C]-amino acids into the proteins of washed cells but no effect in whole semen. Iodoacetate had relatively little effect under aerobic conditions but when incubations were in nitrogen it was an effective inhibitor. Chloramphenicol showed a limited inhibitory effect on both whole semen and washed cells.

p-Fluorophenylalanine, 2:4-dinitrophenol, sodium fluoride and iodoacetate inhibited the motility of spermatozoa whereas the other compounds had

Fig. 5. Effect of cell concentration on the incorporation of [^{14}C]*Chlorella* hydrolysate into the proteins of washed spermatozoa. All incubations were for 2 hr. in a volume of 3 ml., with 0·05 ml. of [^{14}C]*Chlorella* hydrolysate. For details, see text. Specific activities of proteins have not been corrected for radioactivity at zero incubation time. A cell concentration of one unit is equivalent to 80×10^6/ml.

Table 7. *Effect of various metabolic inhibitors and inhibiting conditions on the incorporation of* [^{14}C]Chlorella *hydrolysate into the proteins of bull spermatozoa*

The whole semen (0·3 ml.) or washed cell suspension in fructose–bicarbonate buffer (3 ml.) was incubated with inhibitor and 0·05 ml. of the *Chlorella* hydrolysate as described in the text. Inhibitors were present in the following final concentrations: ribonuclease, 100 μg./ml.; 8-azaguanine, 50 μg./ml. (0·33 mM); chloramphenicol, 50 μg./ml. (0·15 mM); *p*-fluorophenylalanine, 1·83 mg./ml. (10 mM); dinitrophenol, 184 μg./ml. (1 mM); fluoride, 10 mM; iodoacetate, 5 mM. Anaerobic incubations were done under nitrogen, controls were incubated in air. Inhibition (−) or stimulation (+) is given as a percentage of the control at the appropriate time.

		Washed spermatozoa				Whole semen			
		1½ hr.		3 hr.		1 hr.		3 hr.	
Inhibitor	Gas phase	Change in specific activity of proteins (%)	Change in motility (%)	Change in specific activity of proteins (%)	Change in motility (%)	Change in specific activity of proteins (%)	Change in motility (%)	Change in specific activity of proteins (%)	Change in motility (%)
None	O_2	+ 22	None	+ 11	None	—	—	—	—
None	N_2	− 14	None	− 26	None	None	− 40	None	None
Ribonuclease	O_2	None	None	None	None	None	− 17	None	None
8-Azaguanine	O_2	None	− 33	None	None	None	− 17	None	None
Chloramphenicol	O_2	None	− 14	− 23	None	− 35	None	− 27	− 50
p-Fluorophenylalanine	O_2	− 13	− 100	− 26	− 98	None	− 70	None	− 100
Dinitrophenol	O_2	None	− 100	+ 14	− 100	+ 20	− 17	None	− 100
Fluoride	O_2	None	− 86	− 26	− 92	None	− 100	None	− 100
Fluoride	N_2	− 17	− 100	− 10	− 100	None	− 100	None	− 85
Iodoacetate	O_2	None	− 100	− 16	− 100	+ 24	− 100	+ 70	− 100
Iodoacetate	N_2	− 52	− 100	− 52	− 100	− 41	− 100	− 16	− 100

little effect. The inhibiting effect of fluoride and iodoacetate on the motility of spermatozoa has been described earlier (Lardy & Phillips, 1943; Mann & Lutwak-Mann, 1948). Anaerobic conditions did not affect motility, which also is in accord with earlier observations (Ivanov, 1931; Redenz, 1933). In the presence of inhibitors there appeared to be no correlation between the degree of motility and the rate of incorporation of amino acids.

Bacterial contamination

Since bull semen usually contains small numbers of bacteria (Dott & Glover, 1954), it was necessary to preclude the possibility that the incorporation of radioactive amino acids observed in our experiments with spermatozoa was caused by growth of bacteria. In several experiments the number of bacteria present initially, and after various periods of incubation of the washed cell suspension, was determined. The semen of bull N_1 was found to contain about 5000 bacteria/ml., and there was no detectable increase in this number during incubation for periods up to 2 hr. Since 1 ml. of semen contains about 1000 million spermatozoa and since the rate of incorporation was initially fairly high and fell throughout the incubation period, it is very unlikely that the observed incorporation can be due to the small number of bacteria present.

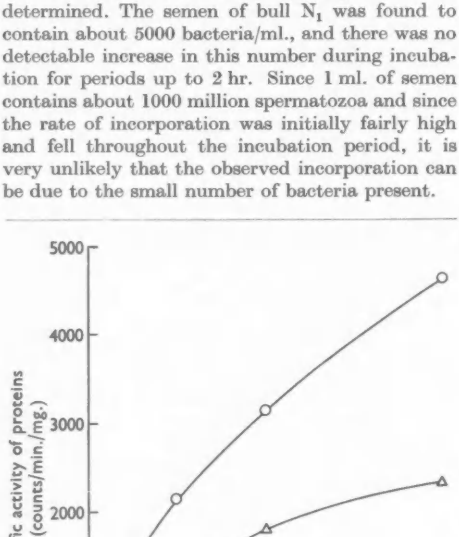

Fig. 6. Incorporation of [^{14}C]*Chlorella* hydrolysate into the proteins of suspensions of rabbit reticulocytes at two levels of cell concentration; ○, 22×10^6 cells/ml. (2·35 mg. of protein/ml.); △, 88×10^6 cells/ml. (9·4 mg. of protein/ml.); 0·1 ml. of [^{14}C]*Chlorella* hydrolysate was added for each 10 ml. of cell suspension. For details, see text.

DISCUSSION

The experiments described in this paper continued over about 1 year. During that time, the rate of amino acid incorporation varied within fairly wide limits but the value derived from nine experiments with washed spermatozoa and [^{14}C]*Chlorella* hydrolysate as source of radioactive amino acid was about 0·035 μmole/g. of protein/hr. This is about one-twentieth of the rate of incorporation obtained with washed reticulocytes under similar conditions. When whole semen was used instead of washed cells, the initial rate of incorporation was greater (after allowance for the weight of inert seminal plasma protein) but incorporation continued for only 20 min. (Fig. 2). We cannot at present offer any explanation of this abrupt cessation of incorporation into the cell fraction of whole semen. With washed spermatozoa and [^{14}C]-valine, the mean rate of incorporation was again between one-tenth and one-twentieth (Table 4) of that obtained with reticulocytes under similar conditions. In whole semen, however, the rate of incorporation of valine was much lower than in

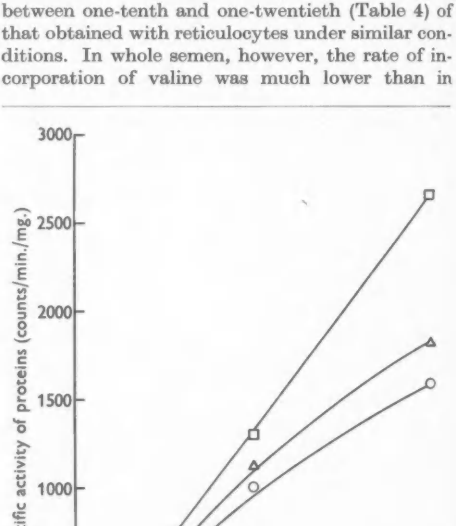

Fig. 7. Incorporation of [^{14}C]valine into the proteins of rabbit reticulocytes at three levels of valine concentration (○, 0·095 mM; △, 0·996 mM; □, 9·94 mM). Each flask contained 0·11 ml. of the [^{14}C]valine solution, the appropriate amount of [^{12}C]valine and 270×10^6 cells in 10 ml. Specific activity values have been adjusted for the dilution by [^{12}C]valine (see text).

washed cells. We are unable to offer any explanation for the failure of spermatozoa in whole semen to incorporate valine at a significant rate.

The amino acid incorporation observed in these experiments is clearly not caused by physical adsorption, since there was no reduction in the specific activity of protein after treatment with either performic acid or sodium hydroxide. Although this indicates that the radioactive amino acids are linked to the protein fraction by covalent bonds, it in no way proves that the process is one of protein synthesis. Additional evidence on the nature of the incorporation process was sought by the use of compounds known to act in other biological systems as effective inhibitors of protein synthesis. Of the inhibitors tried, only iodoacetate under anaerobic conditions produced substantial inhibition of incorporation. The inhibitor tests thus suggest that although the incorporation is a true metabolic reaction, it is not the usual process of protein synthesis. Spermatozoa differ in many respects from other mammalian cells, especially in their nucleic acid, nucleotide and amino acid composition (Bhargava *et al.* 1959); thus the incorporation of amino acids into the proteins of spermatozoa may represent a process not normally detected in other mammalian cells. Carboxyl group activation of amino acids by enzymes from rat liver is not inhibited by either fluoride or by dinitrophenol (Rendi, Di Milia & Fronticelli, 1958). If similar enzymes function in spermatozoa, an inhibitor-resistant amino acid activation could lead to labelling of reactive sites on spermatozoon proteins.

In both spermatozoa and reticulocytes the rate of amino acid incorporation is inversely related to the concentration of cells. In each case the amount of radioactive amino acid incorporated represented a very small fraction of the total amino acids present in the medium. The observed effect of cell concentration cannot therefore be attributed to competition for amino acids. There is, however, a variable dilution of added [^{14}C]amino acid by [^{12}C]amino acids of the cells and also appreciable breakdown of spermatozoon protein during incubation (Bhargava *et al.* 1959). These two factors together could explain the inverse relationship between cell concentration and uptake of [^{14}C]amino acid, but since both respiration and fructolysis of bull spermatozoa are also inversely related to cell concentration the situation is obviously very complex (cf. Bishop & Salisbury, 1955*a*, *b*).

In conclusion, we wish to mention some additional experiments on amino acid incorporation that were carried out with decapitate spermatozoa. The adjective decapitate is used to describe spermatozoa in which the head is disconnected from the tail at the neck (Bishop & Walton, 1959). This condition occurs spontaneously in all the spermatozoa of certain sterile Guernsey bulls (Hancock & Rollinson, 1949; Hancock, 1955). Most of the heads, as judged by their staining reactions, appear to be dead at ejaculation, but most of the tails remain actively motile for several hours *in vitro*. The tails can be separated from the heads by differential centrifuging, and are devoid of either DNA or RNA. Experiments similar to those described earlier in this paper were performed with decapitate spermatozoon tails from three Guernsey bulls. The rates of incorporation of amino acids into protein in these preparations were substantial but with all three bulls the bacterial content of the 'decapitate'-sperm semen was much greater than that of normal bull semen and the interpretation of our results therefore remains equivocal. These results are mentioned here because they have obtained some currency through oral communications and because the 'decapitate' condition is rather rare, and unless bulls can be found producing 'decapitate'-sperm semen which is free of bacteria further progress along these lines will not be possible.

SUMMARY

1. The incorporation of radioactive amino acids into the proteins of bull semen has been measured.

2. In whole semen, the incorporation is exclusively into the spermatozoon protein.

3. In suspensions of washed spermatozoa, incorporation continues for several hours but in whole semen incorporation stops after about 20 min.

4. The incorporation reaction has been compared with that observed in reticulocytes incubated under similar conditions. The rate of incorporation into spermatozoon protein is about one-twentieth of that into reticulocyte protein.

5. Small numbers of bacteria were always present in semen but their presence did not account adequately for the observed incorporation.

6. The rate of incorporation into both spermatozoa and reticulocytes was inversely related to the concentration of cells present.

7. The effect of various inhibitors on the incorporation reaction was examined.

8. It is concluded that the incorporation reaction in spermatozoa is unlikely to be a true measure of protein synthesis.

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Incorporation of ^{65}Zn in the Sub-cellular Fractions of the Liver and Spontaneously Occurring Mammary Tumours of Mice after the Injection of Zinc–Glycine Containing ^{65}Zn

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Relatively little is known about the intracellular distribution of zinc in normal and tumour tissues. Heath (1949) and Heath & Liquier-Milward (1950) obtained evidence that zinc in tumour tissue is associated with nucleoprotein. Their interpretation of the results was, however, complicated by the fact that, since they adopted the method of Dounce (1943), citric acid was used in the isolation of the cell nuclei and it is certain that much or all of the zinc present would be leached out by this acid. Rosenfeld & Tobias (1951) could obtain no evidence for the association of zinc with nucleoprotein in the cell nucleus, but these workers also used a citric acid method (that described by Mirsky & Pollister, 1946) for the isolation of cell nuclei. In investigations on normal rat liver Thiers & Vallee (1957) found that the zinc was present in the nuclear, mitochondrial and 'supernatant' fractions, the lowest concentration being in the mitochondria and the highest in the supernatant and microsomal fractions.

An increased uptake of ^{65}Zn by spontaneous mammary tumours compared with normal mammary tissue was found in female mice injected with the isotope as a zinc–glycine complex (Tupper, Watts & Wormall, 1955). A similar increased uptake of ^{65}Zn has been found in skin tumours induced in mice by 3:4-benzpyrene if the tumours are compared with normal skin (M. E. Bartholomew & R. Tupper, unpublished work). This difference in zinc metabolism between tumours and their normal tissue of origin led us to investigate more fully the binding of zinc in spontaneous mammary tumours of mice, and this paper records the results of our studies on the distribution of injected zinc in the various sub-cellular fractions separated from spontaneous mammary tumours of mice injected with zinc–glycine containing ^{65}Zn. For comparison, similar measurements have been made in the corresponding fractions of the livers of the same mice. The choice of the liver for this purpose was influenced by the fact that there is available a considerable amount of information about the intracellular particles of this organ, and furthermore the liver contains at least two zinc-containing enzymes in which we are particularly interested; at least one of these, glutamic dehydrogenase, is present in the mitochondria.

A preliminary study has also been made on the stability of the zinc compounds in these tissues, and the extent to which the zinc can be removed by prolonged dialysis at different pH values has been

determined. A brief account of some of the results presented here has been reported (Bartholomew, Tupper & Wormall, 1959).

EXPERIMENTAL

Materials

Animals. The mice used were 'pure-line' females of the C3H, ABC and Strong A strains bred in our Laboratory from animals kindly supplied to us by the Institute of Cancer Research (Royal Cancer Hospital) and the Imperial Cancer Research Fund Laboratories. The animals were fed on food pellets (Diet 41, Associated London Flour Millers), oats and water, with a supplement of lard, milk and crushed dog biscuits on 1 or 2 days in each week.

Zinc-free water. This was obtained by passing distilled water through an ion-exchange column containing a mixture, in equal proportions, of De-Acidite FF and Zeo-Karb 225 (The Permutit Co. Ltd.).

Zinc-free NaCl. A solution of NaCl was extracted with a solution of diphenylthiocarbazone (dithizone) in carbon tetrachloride until the dithizone no longer turned red. The NaCl solution was then evaporated to dryness and the 'zinc-free' salt used in the preparation of a 0·9% NaCl solution.

Zinc-free glassware. All glassware used in the dialysis experiments was soaked for at least 24 hr. in 2 N-HCl and then thoroughly washed with zinc-free water.

Buffer solutions. All reagents used in the preparation of buffer solutions were of A.R. grade. Buffers at pH 4·0, 5·6, 6·4, 7·4, 8·4 and 10·0 were prepared according to the tables given by Vogel (1942). Care was taken to ensure that the buffers contained no salt which would form a precipitate with zinc. An acetic acid–sodium acetate mixture was used for buffers at pH 4·0 and 5·6, potassium hydrogen phthalate–potassium sodium phthalate for pH 6·4 and glycine–sodium glycinate for pH 8·4 and 10·0.

Sodium diethylbarbiturate buffer containing ethylenediaminetetra-acetic acid. A 0·02 M-solution of disodium ethylenediaminetetra-acetate EDTA (100 ml.) was diluted to 2 l. with 0·1 M-sodium diethylbarbiturate buffer, pH 7·45. The pH of the final solution was 7·4.

Zinc–glycine solution. $ZnSO_4,7H_2O$ (14·068 g. = 3·2 g. of zinc) and glycine (14·684 g.) were dissolved in water. N-NaOH was added to the solution to bring the pH to 7·4 and water was then added to make a total volume of 4 l. (800 μg. of zinc/ml.). The solution was saturated with chloroform to inhibit the growth of moulds and bacteria.

Radioactive zinc. Radioactive zinc of high specific activity was separated as $ZnCl_2$ from cyclotron-irradiated copper by the method described by Tupper & Watts (1954).

Methods

Preparation of ^{65}Zn *for injection.* The ^{65}Zn was injected as a ^{65}Zn-containing zinc–glycine complex in solution at pH 7·4. The complex was prepared by dissolving glycine (10 mg.) in the stock ^{65}Zn-containing $ZnCl_2$ solution (1 ml.), adding N-NaOH to give a pH of about 7·4 and sufficient water to make the solution iso-osmotic. The final solution contained 6 μg. of zinc/ml. and gave a counting rate of 3×10^6 counts/min./ml. with the scintillation counter used in these investigations (background count rate was 150 counts/min.).

Injection of ^{65}Zn. Mice bearing spontaneously occurring mammary tumours (1–2 cm. diam.) were each given an intraperitoneal injection of 1 ml. of the ^{65}Zn-containing solution.

Removal of tissues. The mice were killed with coal gas 1 or 3 days after injection of the isotope. The tumours and livers were removed as rapidly as possible and placed in beakers chilled to 0–4°.

Determination of ^{65}Zn. The scintillation-counter method described by Banks, Tupper, Watts & Wormall (1955) was used to determine ^{65}Zn.

Total nitrogen determinations. These were made by the micro-Kjeldahl method, the ammonia being distilled in the Markham (1942) apparatus, into a boric acid solution containing methyl red–methylene blue indicator.

Separation of sub-cellular fractions. In some of our earlier experiments a blender (MSE Nelco) was used to prepare tumour and liver mash for the separation of sub-cellular fractions by differential centrifuging. The use of this blender was abandoned in later experiments in favour of a Potter–Elvehjem homogenizer with a Teflon (polytetrafluoroethylene) pestle (A.H.T. Co., Philadelphia, Pa., U.S.A.). Mash prepared with this homogenizer yielded sub-cellular particles showing less damage than those prepared with the blender.

The various cell suspension media investigated were 0·88 and 0·25 M-sucrose and 0·25 M-sucrose containing 7·3% of polyvinylpyrrolidone (Novikoff, 1955). The last-named medium gave good mitochondrial preparations but its nitrogen content was too high to enable us to make accurate allowances for it. We finally chose 0·25 M-sucrose and used the method of separation described by Schneider & Hogeboom (1950).

The centrifuge used was a refrigerated machine with a superspeed attachment (MSE). The speed was not sufficiently high to enable us to separate the microsomes from the supernatant fraction.

Dialysis of mashes. Zinc-free 0·9% NaCl (50 ml.) was added to either the tumours or the livers obtained from four or five mice killed 3 days after the injection of the zinc–glycine containing ^{65}Zn, and mash was prepared in a Waring Blendor.

Samples of the mash were dialysed in sacs made from Visking transparent cellulose tubing. The ratio of the volume of mash to dialysing medium was always 1:2·5. Dialysis was carried out at 0–4° and diffusates were changed twice in each 24 hr. The mashes were dialysed against buffers at pH 4·0, 5·6, 6·4, 7·4, 8·4 and 10·0, and also against solutions at pH 7·4 containing either excess of zinc or buffered EDTA.

At the end of the dialysis period the sacs and their contents were digested with H_2SO_4 and the digests were used for ^{65}Zn determinations; similar determinations were made on samples of the undialysed mash. Recoveries of ^{65}Zn for the dialysed solution plus the corresponding diffusates were good (not less than 95%).

RESULTS

Distribution of ^{65}Zn *between the sub-cellular fractions of mammary tumour and liver tissue*

Microscopic observation of the nuclear fractions showed them to consist mainly of free nuclei and cell debris almost free from whole-cell contamination.

Mitochondria examined under the phase-contrast microscope were mostly spherical in form, although some filamentous granules were also present. The mitochondrial fractions were uncontaminated by cell nuclei.

Fractionations were made on mammary tumour and liver tissue from mice killed 1 or 3 days after the injection of the ^{65}Zn–glycine complex. Each fractionation was made on the pooled tissues from four or five mice. Six fractionations were carried out with the mice killed 1 day after the injection and a similar number with those killed after 3 days. The results given in Tables 1, 2 and 3 are the means for the tissues obtained from 24–30 mice.

The results showing the distribution of ^{65}Zn in the different fractions of the mash can be presented in several ways, and for comparisons of the sort under study here it is often desirable that the results should be calculated in several units or with reference to more than one standard. For example, the ^{65}Zn content of one of the sub-cellular components of a mash can be recorded as a fraction or percentage of the amount of ^{65}Zn present in the whole mash, or all the values may be expressed in terms of ^{65}Zn/mg. of organic nitrogen present in that particular fraction or whole mash. In the latter case, a more accurate picture is presented of the relative zinc/protein ratios. Both methods have been used for Tables 1 and 2, where the results are given for the tissues of mice killed respectively 1 and 3 days after the injection of zinc–glycine.

Another method is to calculate all the results in terms of ^{65}Zn/N, and then to present the results for each mash fraction as the following ratio: ^{65}Zn/N in the fraction, divided by the ^{65}Zn/N value for the original homogenate or mash from which the fraction was separated. This method, which has been adopted for the preparation of Table 3, often allows one to make a more useful assessment of the distribution of the isotope under investigation.

Table 1. *Distribution of* ^{65}Zn *and nitrogen between the intracellular components of mammary tumour and liver tissue after* 1 *day*

Tissues were obtained from mice killed 1 day after the injection of ^{65}Zn as the zinc–glycine complex.

Tables 1–3 record the mean values for each set of fractionations, the mean value being followed by the S.E. and the number of fractionations. Radioactivity results were based on net counts ranging from 4×10^4 to $1{\cdot}5 \times 10^5$ counts/min.; the samples taken for assay gave net counts of 3×10^2–4×10^4 counts/min.

	^{65}Zn (percentage of total ^{65}Zn in whole mash)		N in the fraction (percentage of total N in whole mash)		^{65}Zn (percentage of total ^{65}Zn in whole mash)/mg. of N	
Fraction	Tumour	Liver	Tumour	Liver	Tumour	Liver
Washed nuclei and cell debris	42·07±2·65 (6)	12·88±1·05 (6)	40·51±3·74 (6)	30·20±1·64 (6)	2·37±0·38 (6)	0·60±0·03 (6)
Washed mitochondria	5·47±0·94 (6)	4·54±0·60 (6)	5·85±0·86 (6)	10·64±0·95 (6)	2·01±0·22 (6)	0·58±0·04 (6)
Supernatant (containing microsomes and soluble proteins)	41·04±1·31 (6)	67·43±2·06 (6)	36·74±1·23 (5)	37·40±0·87 (6)	2·52±0·34 (5)	2·55±0·20 (6)
Whole mash	—	—	—	—	2·19±0·21 (6)	1·40±0·09 (6)

Table 2. *Distribution of* ^{65}Zn *and nitrogen between the intracellular components of mammary tumour and liver tissue after* 3 *days*

Tissues were obtained from mice killed 3 days after the injection of ^{65}Zn as the zinc–glycine complex. See note in Table 1.

	^{65}Zn (percentage of total ^{65}Zn in whole mash)		N in the fraction (percentage of total N in whole mash)		^{65}Zn (percentage of total ^{65}Zn in whole mash)/mg. of N	
Fraction	Tumour	Liver	Tumour	Liver	Tumour	Liver
Washed nuclei and cell debris	35·20±3·34 (6)	17·31±1·49 (6)	40·06±2·97 (6)	31·8±1·28 (6)	1·86±0·32 (6)	0·78±0·11 (6)
Washed mitochondria	6·41±0·38 (6)	9·54±0·81 (6)	6·03±0·31 (6)	15·92±1·18 (6)	2·17±0·16 (6)	0·84±0·15 (6)
Supernatant (containing microsomes and soluble proteins)	45·53±2·59 (6)	59·44±2·34 (6)	45·11±2·86 (6)	37·44±1·06 (6)	2·21±0·47 (6)	2·20±0·24 (6)
Whole mash	—	—	—	—	2·13±0·36 (6)	1·38±0·14 (6)

Table 3. ^{65}Zn *content of the intracellular components of mammary tumour and liver tissues from mice injected with* ^{65}Zn *as the zinc–glycine complex (calculated as a special ratio)*

See note in Table 1.

^{65}Zn content [ratio $\frac{(^{65}\text{Zn/mg. of N in fraction})}{(^{65}\text{Zn/mg. of N in whole mash})}$]

Fraction	1 day after injection		3 days after injection	
	Tumour	Liver	Tumour	Liver
Washed nuclei and cell debris	1·06±0·07 (6)	0·43±0·03 (6)	0·87±0·04 (6)	0·55±0·04 (6)
Washed mitochondria	0·92±0·07 (6)	0·42±0·02 (6)	1·08±0·08 (6)	0·60±0·04 (6)
Supernatant (microsomes and soluble proteins)	1·10±0·04 (5)	1·82±0·09 (6)	1·02±0·06 (6)	1·59±0·05 (6)
Whole mash	1·00	1·00	1·00	1·00

^{65}Zn was found in all three sub-cellular fractions (nuclei, mitochondria and supernatant) of both mammary tumours and liver. The distribution pattern in the tumour was, however, very different from that of the liver. From Table 3 it may be seen that the three fractions of the tumour cells have about the same ^{65}Zn concentration. In the mice killed 1 day after injection, the lowest ratio was found in the mitochondria (mean of 0·92) and the highest in the 'supernatant' fraction (1·10). The ratios for the three tumour fractions were very similar for the mice killed 3 days after the injection, though a rather low value (mean of 0·87) was obtained for the nuclear fraction.

One day after the injection most of the ^{65}Zn in the tumour was divided almost equally between the nuclei and the supernatant, with about 40 % in each. Three days after the injection, rather more ^{65}Zn (45 %) was found in the supernatant than in the nuclei (35 %).

In the liver, most of the ^{65}Zn was present in the supernatant fraction 1 and 3 days after the injection. In the liver, the mitochondrial fraction contained the lowest amount of ^{65}Zn of the three fractions studied.

Dialysis of mammary tumour and liver mash

In order to study the firmness of the binding of the zinc to the proteins or other constituents of the tissues, we have determined the extent to which the ^{65}Zn can be removed from tumour and liver mash when the mash is dialysed for a long time in solutions of different pH values. In some experiments made at pH 7·4, a considerable amount of zinc (as zinc–glycine) was added to the dialysing medium to determine whether this extra zinc would effect removal of ^{65}Zn from the tumour or liver by exchange; in other experiments the strong metal-binding or chelating agent EDTA was added to the dialysing medium.

The results showing the means for duplicate experiments are given in Table 4. At pH 7·4 no ^{65}Zn dialysed from tumour or liver mash except when EDTA or excess of zinc was present in the dialysing medium. In the pH range studied (pH 4–10) greatest removal of zinc occurred at pH 4·0, at which acidity about 90 % of the tissue-bound ^{65}Zn dialysed out from both tumour and liver mash within 96 hr. In all these dialyses, except those at pH 7·4 (where none of the ^{65}Zn was removed by dialysis) and at pH 6·4, more zinc was firmly bound in the liver mash than in the tumour tissue.

Table 4. *Effect of* pH *on the binding of* ^{65}Zn *in mouse mammary tumour and liver mash obtained from tissues labelled* in vivo

pH	^{65}Zn remaining in mash after dialysis for 96 hr. (percentage of ^{65}Zn originally present in mash)	
	Liver	Mammary tumour
4·0	10·2	7·7
5·6	62·0	42·7
6·4	76·6	94·4
7·4	100·0	100·0
7·4*	40·2	31·7
7·4†	30·8	28·1
8·4	74·8	60·0
10·0	52·2	47·4

* EDTA added to buffer.
† Zinc added to buffer.

When excess of zinc or the zinc-binding EDTA was added to the dialysing medium and the dialysis carried out at pH 7·4, 60–70 % of the tissue zinc, which is completely non-dialysable at this pH, can be readily removed by dialysis for 96 hr. The zinc remaining after this period dialysed extremely slowly and about 15 % of the original ^{65}Zn remained in the mash dialysed for 400 hr. against excess of zinc or for 670 hr. against buffer containing EDTA. Both tumour and liver mash contained this very firmly bound ^{65}Zn.

DISCUSSION

The results summarized in Tables 1, 2 and 3 show that there was a remarkably uniform distribution of ^{65}Zn between the three sub-cellular fractions of the tumour cells of the mice injected with zinc–glycine containing ^{65}Zn. The lowest concentration of ^{65}Zn (per mg. of nitrogen) in the tumours of the mice killed 1 day after the injection was found in the mitochondria, and the highest in the supernatant. By the third day, the ^{65}Zn contained in the mitochondria had risen slightly and that of the supernatant had fallen, but in general the differences between the ^{65}Zn concentrations of the three tumour sub-cellular fractions examined were not marked. This uniform distribution of ^{65}Zn between the three tumour fractions, and the high concentration of ^{65}Zn in the liver 'supernatant' compared with that in the liver nuclei and mitochondria, are well illustrated (as in Table 3) when the ^{65}Zn concentration in the separated fractions is assessed by the 'ratio' (^{65}Zn/mg. of nitrogen in the fraction)/(^{65}Zn/mg. of nitrogen in whole mash).

The distribution pattern of ^{65}Zn in the liver was quite different from that in the tumour. In the liver the ^{65}Zn concentrations of the nuclear and mitochondrial fractions were equal and approximately a quarter or a third that of the supernatant fraction. The ^{65}Zn content of the mitochondria and nuclei 3 days after the injection was higher than that 1 day after injection, but the incorporation of zinc into these fractions of the liver is a comparatively slow process.

Incorporation of ^{65}Zn into the tumour-mitochondrial and -nuclear fractions was considerably faster than into the corresponding liver fractions. The ^{65}Zn content of the tumour mitochondria was little greater 3 days after the injection than at the end of the 1-day period; in comparison, the concentration of ^{65}Zn/mg. of nitrogen in the liver mitochondria on the third day after the injection was one and a half times that at the 1-day period.

Whether the uniform distribution of ^{65}Zn between the various tumour fractions is typical of the tumour and not of the normal gland is unknown. The fractionation of normal mouse mammary-gland tissue into its sub-cellular components is very difficult because of the large amount of fat present and the small amount of tissue available, but the results of our preliminary experiments indicate that in the normal mammary gland of mice injected with zinc–glycine containing ^{65}Zn, the intracellular distribution of ^{65}Zn differs significantly from that in the mammary tumours.

From the results of the dialysis experiments, certain tentative conclusions can be reached. The ^{65}Zn in the tissues studied was mainly in a form in which it was readily exchangeable with Zn^{2+} ions, or removable by the chelating agent EDTA. Part of the zinc in both the tumour cells and the liver cells can also be readily removed by dialysis in slightly acid or alkaline solutions. For example, at pH 5·6 there was a loss of 40–60% of the ^{65}Zn when these tissues were dialysed for 96 hr. At pH 6·4, however, about 95% of the ^{65}Zn in the tumour, and 75% in the liver mash, was not dialysable under these conditions, and it may be significant that the pK for imidazole groups in a typical protein (serum albumin) is 6·3 (Schubert, 1954). A considerable amount of evidence is available that imidazole groups have a strong affinity for zinc and, in serum albumin at least, the intrinsic affinity constant agrees well with that for simple zinc–imidazole complexes (Gurd & Goodman, 1952). The results of the dialysis experiments reported here, and other evidence we have obtained (unpublished work), are consistent with the view that much of the ^{65}Zn in these tissues and others we have studied is bound to imidazole groups of the proteins. When these tissues are dialysed at about pH 7·4 against a solution of zinc–glycine in which complex the zinc is present in a readily 'available' or exchangeable form (Tupper, Watts & Wormall, 1951), nearly all the zinc present as ^{65}Zn in both the liver and tumour tissues is readily removed. About one-sixth of the ^{65}Zn in these tissues is, however, very firmly bound and cannot be removed by long dialysis in the presence of excess of zinc–glycine or the metal-binding EDTA. In all probability this ^{65}Zn is present in zinc-containing enzymes or other zinc proteins in which the metal is an integral part of the molecule or in both.

SUMMARY

1. The distribution of ^{65}Zn in the nuclear, mitochondrial and 'supernatant' (soluble proteins plus microsomes) fractions of mammary tumours and livers of mice, 1 and 3 days after the mice have been injected with the isotope in the form of a ^{65}Zn-containing zinc–glycine complex, has been studied. The results are expressed as ^{65}Zn/mg. of nitrogen in any particular fraction, and also as a special ratio: (^{65}Zn/nitrogen in the sub-cellular fraction)/(^{65}Zn/nitrogen in the whole mash).

2. ^{65}Zn was found in nuclear, mitochondrial and supernatant fractions of mammary tumours and livers.

3. The tumour cells showed a remarkably uniform distribution of ^{65}Zn between the three fractions, but the liver supernatant fractions contained three or four times as much ^{65}Zn (per mg. of nitrogen) as did the nuclei and mitochondria.

4. Part of the ^{65}Zn incorporated into these tissues can be removed by dialysis in slightly acid or alkaline solutions but not normally at pH 7·4.

The results of these dialyses are consistent with the view that the zinc is mainly bound to imidazole groups of the proteins.

5. About four-fifths of the ^{65}Zn present in both the liver and the tumour is freely exchangeable with Zn^{2+} ions at pH 7·4 and can be removed by dialysing the mash against buffers containing excess of exchangeable zinc or the zinc-binding ethylenediaminetetra-acetic acid.

6. About one-sixth of the ^{65}Zn in these tissues could not be removed by exchange or by combination with ethylenediaminetetra-acetic acid, and this fraction probably represents ^{65}Zn in zinc bound to protein.

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Incorporation of Canavanine by *Staphylococcus aureus* 524 SC

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Previous experiments (Richmond, 1959) have shown that *Staphylococcus aureus* 524, which is normally exacting for arginine, can, when incubated in a fully synthetic amino acid medium containing canavanine in place of arginine, increase in turbidity and synthesize cell protein. Under these conditions, however, the cultures do not synthesize active forms of β-galactosidase, hyaluronidase, phosphatase or the lytic enzyme. It seemed possible therefore that canavanine was incorporated into cell protein in place of arginine, resulting in the formation of inactive enzymes.

A number of workers have reported that certain amino acid analogues can be incorporated into protein of micro-organisms in place of normal amino acids and that this process is associated with variable effects on the enzyme-forming ability of the organisms (Munier & Cohen, 1956, 1959; Cowie & Cohen, 1957; Pardee & Prestidge, 1958; Cohen & Munier, 1959).

This paper describes the detection of canavanine in the protein of *S. aureus* which has been grown in its presence.

MATERIALS AND METHODS

Organism. Staphylococcus aureus 524 SC (Rogers, 1953) has been used throughout these studies. The organism was maintained as described by the author (Richmond, 1959).

Media. Hedley–Wright broth (Rogers, 1954) and the fully synthetic amino acid media 18AA and 17AA(-Arg) have been described previously (Richmond, 1959). Experiments involving the use of radioactive lysine were carried out in 18AA medium from which the lysine had been omitted (17AA-Lys medium).

Amino acids and derivatives. The compounds used in these studies and their sources were as follows. L-Canavanine (Dr J. Mandelstam and Dr R. Davies); dinitrophenyl (DNP)-arginine (Dr F. Sanger); DNP-ε-lysine (Dr R. R. Porter). The [1-^{14}C]lysine was obtained from The Radiochemical Centre, Amersham, Bucks. It was used at a specific activity of 0·1 μc/m-mole.

Preparation of organisms for experimental purposes. Unless otherwise stated, organisms were prepared as described previously (Richmond, 1959) and inoculated into 17AA(-Arg) medium containing 50 μg. of canavanine/ml. The culture was incubated at 35° and shaken at the maximum rate (87 cyc./min., 1 ft. throw) of a graded shaker (Kantorowicz, 1951); growth was followed turbidimetrically

(Rogers, 1954) and samples were removed for fractionation when required.

Cell fractionation. Samples were precipitated by the addition of sufficient cold trichloroacetic acid (20%, w/v) to bring the final concentration to 5%. After standing for 2 hr. at 2°, the precipitate was removed on a centrifuge and fractionated to yield the ethanol-soluble protein and protein + cell wall fraction (Richmond, 1959). Cell wall preparations were made by the method devised by Cummins & Harris (1956) and modified by Mandelstam & Rogers (1959).

Preparation of dinitrophenyl-protein (DNP-protein). About 20 mg. of protein + cell wall fraction was suspended in 0·4 M-trimethylamine carbonate buffer, pH 9·0, and treated with 1-fluoro-2:4-dinitrobenzene (FDNB) as described by Sanger & Thompson (1953). Excess of reagent and dinitrophenol were removed as described by those authors.

Hydrolysis of normal and dinitrophenyl-protein + cell wall fractions. Samples were hydrolysed overnight in 6 N-HCl at 105° in sealed tubes to liberate free amino acids. At the end of this period, the hydrolysates were evaporated to dryness at 100° on a water bath in a stream of air and traces of HCl removed by repeated evaporation of three to four batches (2 ml.) of water at 100°. A sample of 200 μg. of L-canavanine treated in this way was recovered in greater than 80% yield.

Chromatography. Descending chromatography of free amino acids and their DNP derivatives was carried out in either butanol–acetic acid–water (60:10:27, by vol.) or 2-methylbutan-2-ol–0·1 M-phthalate buffer, pH 6·0 (Blackburn & Lowther, 1951). Whatman no. 1 or no. 3 paper was used, depending on the quantity of material to be separated. The detection of the position of bands to be eluted and the method of elution were carried out as described by Mandelstam (1958).

Ionophoresis. Amino acids were separated by ionophoresis on Whatman no. 3 paper in buffers of the following composition: ammonium carbonate (2%, w/v) adjusted to pH 9·1 with aq. NH_3 soln. (sp.gr. 0·880); pyridine–acetic acid–water (10:0·4:90, by vol.), pH 6·5 (Ryle, Sanger, Smith & Kitai, 1955), and pyridine–acetic acid–water (1:10:89, by vol.), pH 3·6 (Ryle *et al.* 1955). All these buffers were removed by heating papers in an incubator at 50° for 30 min.

Most experiments were carried out by the 'closed-strip method' (Block, Durrum & Zweig, 1958), in an apparatus similar to that designed by Kunkel (1954), with a potential gradient of 15 v/cm. The preliminary experiments on the separation of canavanine from the protein of *S. aureus* were done in the apparatus of Ryle *et al.* (1955) with a potential gradient of 40 v/cm. The positions of amino acids on chromatograms and ionophoresis papers were determined with ninhydrin in the normal manner; arginine and canavanine were also detected by the alkaline α-naphthol–diacetyl reagent of Szafir & Bennett (1953).

RESULTS

A culture of *S. aureus* prepared as described above was inoculated to an initial density of about 0·15 mg. dry wt. of bacterial cells/ml. in 17 AA(-Arg) medium containing 50 μg. of L-canavanine/ml. and incubated on a shaker at 35°. A similar culture incubated in 17 AA(-Arg) medium to which 4 μg. of L-arginine/ml. had been added acted as a control. After incubation for 3 hr., the cultures were precipitated with cold trichloroacetic acid and fractionated to yield the protein + cell wall fraction (see Methods section). This fraction, which usually constituted about 50% of the total dry weight of material precipitated, was washed in aqueous 75% ethanol and hydrolysed in 6 N-HCl to liberate free amino acids.

The arginine and canavanine were separated from the other amino acids in the hydrolysates by chromatography with butanol–acetic acid–water with arginine and canavanine markers. Under these conditions, arginine and canavanine run slowly ($R_F \sim 0{\cdot}1$) and do not separate. After 24 hr. in this solvent the chromatograms were dried and the position of the arginine–canavanine band was detected by spraying the markers with ninhydrin. The area containing these amino acids was cut from the chromatogram and eluted into 4 ml. of water. Material separated in this way was usually contaminated with variable quantities of glutamic acid, lysine, proline, histidine and aspartic acid. The eluate was taken to dryness at 100° and transferred to paper for ionophoresis at pH 9·1, 6·5 or 3·5 in the appropriate buffers. A distinct spot corre-

Table 1. *Mobilities of arginine and canavanine during paper electrophoresis in buffers at* pH 9·1, 6·5 *and* 3·5

+ Migration towards cathode; −, migration towards anode.

Buffer	pH value	Distance moved/2 hr. at 40 v/cm. (cm.) Arginine	Canavanine
Ammonium carbonate (2%, w/v)	9·1	+ 5·3	− 6·7
Pyridine–acetic acid–water (10:0·4:90, by vol.)	6·5	+31	+27·5
Pyridine–acetic acid–water (1:10:89, by vol.)	3·5	+43	+43

sponding to canavanine was obtained in the hydrolysate from organisms grown with canavanine at both pH 9·1 and 6·5; no canavanine was detected in the control. At pH 3·5 the arginine and canavanine markers did not separate and no additional spot was detected in hydrolysates of organisms grown in the presence of the analogue. The mobilities of arginine and canavanine on ionophoresis at pH 9·1, 6·5 and 3·5 are shown in Table 1.

Although these results showed that canavanine could be detected in the protein + cell wall fraction of *S. aureus* grown in the presence of the analogue it was possible that the canavanine was adsorbed on to the protein + cell wall fraction rather than incorporated into the protein with formation of covalent bonds. To reduce the possibility that the presence of canavanine was due to adsorption, the following control experiments were carried out.

(1) The efficiency of the fractionation procedure in freeing the protein + cell wall fraction of uncombined basic amino acids was tested as follows. A culture of *S. aureus*, prepared in the normal manner, was suspended to an initial density of 200 μg./ml. in 17AA(-Lys) medium to which [1-^{14}C]lysine had been added. This amino acid was chosen as [^{14}C]arginine was not available. The culture was immediately precipitated with 20% trichloroacetic acid. The precipitate obtained in this way was fractionated by the normal procedure and the contamination of the protein + cell wall fraction with lysine estimated by determining the radioactivity in the sample. The maximum level of radioactivity detected in such experiments showed the contamination of the protein + cell wall fraction to be less than 10 μg. of lysine/g. dry wt. of organisms. A contamination of this magnitude in 20 mg. of protein + cell wall fraction would not be detected by the methods which have been used to detect canavanine.

(2) A culture grown with arginine was precipitated with trichloroacetic acid immediately after the addition of 100 μg. of canavanine/ml. and fractionated as described above. No canavanine could be detected in the protein + cell wall fraction.

(3) A culture was incubated for 3 hr. in 17AA(-Arg) medium to which 50 μg. of canavanine and 50 μg. of arginine/ml. had been added; a culture in 18AA medium acted as control. Under these conditions the organisms grew exponentially at the same rate as the control and no canavanine could be detected in the protein + cell wall fraction of either culture.

(4) Samples of 20 mg. of protein + cell wall fraction from cultures grown in canavanine were subjected to ionophoresis at pH 6·5 and 9·1 before hydrolysis. No canavanine could be detected. Similar samples, separated after hydrolysis, were found to contain canavanine.

(5) Samples of 20 mg. of protein + cell wall fraction from organisms grown with canavanine were converted into the DNP derivative by the action of FDNB. DNP-Protein + cell wall fraction was subjected to ionophoresis at pH 6·5 and 9·1 before hydrolysis. If free canavanine were adsorbed on to the protein the detection of DNP-canavanine would be expected. Dinitrophenol and a little DNP-ϵ-lysine were the only compounds detected. A further sample from the same batch of material, hydrolysed with acid and then separated by ionophoresis, was shown to contain free canavanine. It seems likely therefore that the canavanine found in the protein + cell wall fraction under these conditions is not adsorbed non-specifically but is combined in such a manner that the α-NH_2 group of the molecule is not available to react with FDNB.

In an attempt to obtain an estimate of the quantity of canavanine incorporated into the protein + cell wall fraction during incubation for 3 hr., the arginine and canavanine spots after treatment with ninhydrin were cut from ionophoresis papers run at pH 9·1 and eluted into water. The marker quantities of arginine and canavanine were treated similarly and the quantity of the amino acid was measured by determining the absorption at 570 mμ due to the ninhydrin colour in the eluates. The level of canavanine in the protein + cell wall fraction was found to be about one-tenth of the arginine on a molar basis.

It was not possible to detect either arginine or canavanine in hydrolysates of 50 mg. of cell wall prepared from organisms grown in the presence of the analogue. The canavanine isolated from the protein + cell wall fraction must therefore have originated in the protein part of the fraction.

DISCUSSION

The results reported in this paper suggest that canavanine is incorporated into the protein fraction of *S. aureus* 524 which had been grown in its presence. This strain of *S. aureus* increased in turbidity by about 80% during incubation for 2–3 hr. in a medium containing canavanine but no arginine (Richmond, 1959). Volcani & Snell (1948) and Muraoka & Takadi (1954) have reported that certain *Lactobacillus* species and strains of *Streptococcus faecalis*, which are exacting for arginine, grew to a limited extent when incubated in a medium containing canavanine in place of arginine. A growth response of this kind may be characteristic of bacteria which are capable of incorporating canavanine into their protein.

Despite the incorporation of canavanine into the protein fraction of *S. aureus* 524, it is unlikely that canavanine is incorporated into the protein of all

species of micro-organisms. Pardee & Prestidge (1958) have reported that canavanine is not capable of supporting growth of an arginine-requiring mutant of *Escherichia coli*. This is, perhaps, because some strains of *E. coli* are reported to decarboxylate canavanine to yield an amine containing an *O*-guanidyl group (presumably, $H_2N{\cdot}C(:NH){\cdot}NH{\cdot}O{\cdot}[CH_2]_3{\cdot}NH_2$) (Hagihara, 1956). Furthermore, Kihara, Prescott & Snell (1955) have shown that canavanine can be degraded to homoserine + guanidine by some strains of *Strep. faecalis* and that these strains are therefore resistant to canavanine. Other actions of bacteria on canavanine are summarized by Kalyankar, Ikawa & Snell (1958).

As canavanine is so similar in structure to arginine and active in so many enzyme systems which are normally active on that amino acid (see Kihara *et al.* 1955; Meister, 1957) it is not, perhaps, surprising that it should be incorporated into the protein fraction of organisms. The results reported in this paper give no indication whether canavanine replaces arginine residues in the protein. If such replacement does occur, however, the marked difference between the pH value of the isoelectric point of the two amino acids (arginine, 10·7; canavanine 7·9; Spector, 1956) gives rise to the hope that the 'analogous' proteins may be separated readily by physicochemical methods.

SUMMARY

1. The presence of canavanine was detected in the protein + cell wall fraction of *Staphylococcus aureus* 524 grown in its presence.

2. No canavanine could be detected in cell-wall preparations from cells grown in the presence of arginine under similar conditions.

3. The canavanine was found to be incorporated in the protein + cell wall fraction in such a way that the amino group of the amino acid was not free to react with 1-fluoro-2:4-dinitrobenzene.

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Permeability of *Escherichia coli* to Ribose and Ribose Nucleotides

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Lutwak-Mann (1936) and Stephenson & Trim (1938) have shown that washed suspensions of *Escherichia coli* are able to degrade adenine nucleotides to form hypoxanthine, inorganic phosphate, ammonia and ribose. Stephenson & Trim also found that ribose did not accumulate but was fermented with the liberation of a gas, and that the rate of gas production from adenosine was much greater than that from free ribose.

In previous experiments on the effect of nucleotides on various enzyme reactions in *E. coli* (Eggleston, 1957, 1958; Krebs, Jensen & Eggleston, 1958),

it was noted that one of seven strains of *E. coli* (National Collection of Industrial Bacteria 8571; see Knivett, 1954), when grown on glucose, decomposes anaerobically adenosine 5′-phosphate with the liberation of gas. The gas production proved to be due to the fermentation of the ribose moiety of adenosine 5′-phosphate. Free ribose and ribose 5′-phosphate were not fermented, as was the case with the organism studied by Stephenson & Trim. Ribose and ribose 5′-phosphate were, however, decomposed when the cells were grown in the presence of ribose. This paper is concerned with an investigation of the factors which affect the fermentation of ribose and related substances. The results indicate that the penetration of ribose and ribose 5′-phosphate into the cells is controlled by a mechanism which possesses all the criteria of a permease (Cohen & Monod, 1957).

EXPERIMENTAL

Materials

Nucleotides and nucleosides. Adenosine triphosphate (ATP), sodium salt, and adenosine diphosphate (ADP), barium salt, were obtained from Schwarz Laboratories Inc., N.Y., U.S.A. The chromatographic technique of Krebs & Hems (1953) showed that contamination with other phosphates was less than 5% (in terms of P). Adenosine 5′-phosphate was obtained from Zellstoff-fabrik Waldhof, Wiesbaden, Germany; yeast adenylic acid, guanylic acid, uridylic acid and cytidylic acid, inosine and guanosine from L. Light and Co. Ltd. Adenosine was supplied by Genatosan Ltd., Loughborough, Leics. Flavin mononucleotide was a free gift from Sigma Chemical Co., Mo., U.S.A.

Other reagents. Adenine, hypoxanthine, and D(−)-ribose were supplied by L. Light and Co. Ltd.; ribose 5-phosphate from Sigma Chemical Co. was found to give a single spot when examined by the paper-chromatographic technique of Eggleston (1954), and the concentrations of solutions of this compound were checked by ribose determination. A HCl-hydrolysate of casein was prepared according to Barton-Wright (1952); the HCl was removed by evaporation *in vacuo*.

Buffers. 2-Amino-2-hydroxymethylpropane-1:3-diol (tris)–maleate buffer, pH 6·8, 0·5 M-potassium salt, was made according to Gomori (1955). Phosphate buffer, pH 6·8, M-sodium salt, was made by mixing equal quantities of M-Na_2HPO_4 and M-NaH_2PO_4 solutions. The pH of these buffers at the concentrations used in the experiments was checked with a glass electrode and a Pye pH meter.

Micro-organisms. E. coli strains 8571, F, 86, 8114, 4, T and M/48, were described previously (Eggleston, 1957).

Methods

Suspensions of *E. coli* were grown semi-anaerobically in round flat-bottomed 1 l. flasks filled to the neck and washed as previously described (Eggleston, 1957), except that in some experiments (where stated) the liquid growth medium contained 1% (w/v) of D(−)-ribose instead of the usual 2% (w/v) of glucose. The washed cells were stored at 0° as suspensions in water (about 30 mg. dry wt./ml.).

Whole cells (8–15 mg. dry wt.) were incubated in Warburg vessels in a total volume of 2 ml. This included 0·2 ml. of 0·5 M-tris–potassium maleate buffer, pH 6·8, or variable quantities of M-sodium phosphate buffer, pH 6·8, and 0·1 ml. of 0·1 M-$MgCl_2$ in the main compartment and substrate (usually 0·1 ml. of 0·05 M) in the side bulb. The gas space was filled with N_2 and the temperature of incubation was 25°. The gas evolved on addition of the substrate usually consisted of CO_2 and H_2. In order to measure the proportions of these two gases two vessels (of nearly identical volume) were used, the centre well of one of which contained 0·2 ml. of 2 N-NaOH, freshly prepared, soaked into a pleated filter paper to absorb CO_2. After deduction of the H_2 evolved (in mm.), CO_2 evolution in the vessel without NaOH was calculated with the correction for CO_2 retention at pH 6·8 according to Johnson (1951).

At the end of the incubation period the cup contents were deproteinized by the addition of 0·5 ml. of 30% (w/v) trichloroacetic acid, followed by centrifuging. The clear supernatant solutions were stored at −15° until required for analysis. Paper chromatography was carried out on 50 µl. samples according to Krebs & Hems (1953). When chromatograms were to be examined in u.v. light phosphate buffer was used in place of tris–maleate buffer as the latter absorbed in the u.v. Inorganic phosphate was determined in the trichloroacetic acid extracts by the method of Berenblum & Chain (1938) as modified by Bartley (1953). Ammonia determinations were carried out by distillation in Conway units followed by nesslerization. Ribose was determined by the colorimetric method of Mejbaum (1939), acetic acid by the method of Serlin & Cotzias (1955) and ethanol by the method of Williams, Linn & Zak (1958), except that in place of Conway units the distillation vessels described by Bartley (1953) were used. Succinic acid was determined according to Krebs (1937) and malic acid according to Hummel (1949).

RESULTS

Degradation of nucleotides by whole cells

Washed cells of *E. coli* 8571, when incubated anaerobically at pH 6·8 and at 25°, evolved gas from ATP, ADP, adenosine 5′-phosphate, adenosine

Table 1. *Comparison of the rate of fermentation of various ribose-containing compounds by whole cells of* Escherichia coli 8571

The system contained: 12·4 mg. dry wt. of whole cells; 0·05 M-tris–potassium maleate buffer, pH 6·8; 5 mM-$MgCl_2$; substrates 2·5 mM; final vol. was 2 ml. Gas phase N_2. Temp. 25°.

Substrate	$CO_2 + H_2$ liberated in the first hr. (µmoles)
None	0·51
Ribose	0·80
Ribose 5-phosphate	0·71
ATP	2·21
ADP	3·10
Adenosine 5′-phosphate	6·92
Adenosine	7·05
Inosine	8·08

Table 2. *Degradation of various nucleotides and their derivatives by whole cells of* Escherichia coli 8571

The system contained 16 mg. dry wt. of whole cells; other details were as given in Table 1. After adding the substrate (5 μmoles) from the side arm, the manometers were read until gas evolution from the nucleosides had ceased (90 min.).

Expt. no.	Substrate	$CO_2 + H_2$ liberated (μmoles)	NH_3 formed (μmoles)	Inorganic phosphate formed (μmoles)
1	None	1·92	1·58	2·18
	Adenine	1·88	3·04	—
	Yeast adenosine monophosphate	9·20	5·54	7·03
	Inosine	10·36	1·27	—
	Adenosine	10·52	5·80	—
	Adenosine 5′-phosphate	10·40	5·70	7·68
	Guanosine	11·15	3·04	—
2	None	1·16	1·58	2·18
	Flavin mononucleotide	1·03	2·17	3·08
	Hypoxanthine	1·69	1·58	2·32
	Guanylic acid	6·70	2·66	5·70
	Uridylic acid	7·28	1·20	5·42
	Cytidylic acid	7·68	4·60	5·40
	Inosine	9·15	1·27	2·10

and inosine, but not from ribose and ribose 5-phosphate (Table 1). CO_2 and H_2 were usually formed in equal proportions, though in some experiments CO_2 prevailed in the proportions of 3:2. The fermentation rate was highest with inosine, slightly less high with adenosine and adenosine 5′-phosphate and still lower with ADP and ATP. Gas evolution usually ceased just before 2 μmoles/μmole of substrate added was reached.

The gas evolution from other nucleotides, nucleosides and related substrates is shown in Table 2. Yeast adenylic acid, guanylic acid, uridylic acid, cytidylic acid and guanosine were all fermented, whereas no gas was formed from purines and flavin mononucleotide. Thus fermentable compounds were those in which a ribose molecule was in combination with either a purine or pyrimidine base. Phosphate-containing substrates all liberated inorganic phosphate. There was a release of approximately 1 mol.prop. of NH_3 from adenosine 5-phosphate and adenosine and of less from adenine, guanosine, guanylic acid and cytidylic acid.

After cessation of the gas evolution from the nucleosides no ribose could be detected in the suspensions. When free ribose or ribose 5-phosphate was added their concentrations remained unchanged on incubation. Hypoxanthine was identified by paper chromatography as an end product of inosine and adenine nucleotide degradation. When ATP was the added substrate chromatography showed the intermediate formation of ADP; no adenosine 5′-phosphate or adenosine were detectable but hypoxanthine appeared together with some NH_3 and inorganic phosphate, and the total ribose content decreased approximately in proportion to the gas produced; e.g. during incubation for 70 min. 4·45 μmoles of gas were liberated and 2·21 μmoles of the total ribose disappeared.

There was a major deficit in the balance sheet of the fermentation of the ribose. Thus on incubation with inosine, the carbon of the ribose moiety was recovered as follows: 15–19 % as CO_2, 15–25 % as acetic acid, 15–20 % as ethanol, 10–15 % as succinic acid, 1·5–2·0 % as malic acid; 19–44 % remained unaccounted for. No lactic acid (method of Barker & Summerson, 1941) could be detected. A search for other phosphorylated end products (Krebs & Hems, 1953) or for carboxylic acids (Buch, Montgomery & Porter, 1952) by paper chromatography was unsuccessful.

The rate of degradation of nucleosides, as measured by gas production, was not increased by the addition of inorganic phosphate (0·005–0·05 M). Higher concentrations of phosphate (0·1 M) were slightly inhibitory, and arsenate (0·025 M) completely inhibited gas production from inosine. Omission of K^+ and Mg^{2+} ions did not affect the rates of gas production from nucleosides. Iodoacetate (0·01 M) caused complete inhibition by gas production.

Cells of *E. coli* which have been frozen and thawed repeatedly are known to have an increased permeability to amino acids and nucleotides (Eggleston, 1957). This treatment did not alter their permeability to free ribose or ribose 5-phosphate, but the rates of fermentation of nucleotides and nucleosides were increased by about 60 %.

No appreciable gas production could be detected when whole cells of other *E. coli* strains (F, 4, 8114, 86, M/48 and T) were incubated with adenosine 5′-phosphate. ATP, ADP, adenosine, guanylic acid, uridylic acid and cytidylic acid also gave negative results with strains F and 4.

Fermentation of ribose, ribose 5-phosphate and inosine in cell-free preparations

As the differences in the fermentability of nucleosides on the one hand and of free ribose and ribose 5-phosphate on the other could be due to permeability barriers, the fermentation of these substances was examined in disrupted cells. Disruption of cell suspensions by shaking with Ballotini beads (Mickle, 1948) and centrifuging gave clear extracts incapable of fermenting nucleoside; suspensions of acetone–ether-dried cells (Gale & Epps, 1944) were also inactive. Subjection of a cell suspension (310 mg. dry wt.) in water (10 ml.) to supersonic vibration (at 25 kcyc./sec., generated by a 600 w Mullard magnetostrictor oscillator at 3·5 A) for 3 min. followed by centrifuging gave a clear supernatant with no nucleoside-fermenting activities, and a small sediment with a trace of fermenting power. Combination of sediment and supernatant gave a suspension capable of fermenting inosine, ribose 5-phosphate and free ribose (Table 3). The rate of gas production from inosine in this preparation was about 20 % of that found in whole cells. There was a lag period of over 1 hr. before the fermentation of free ribose began, but incubation of whole cells with ribose under the same conditions failed to lead to an appreciable gas production.

Table 3. *Fermentation of ribose, ribose 5-phosphate and inosine in a cell-free preparation obtained by supersonic vibration*

The sediment and supernatant extracts were prepared and combined as described in the text; each cup contained an amount of enzyme preparation equivalent to about 30 mg. dry wt. of cells; other details were as given in Table 1.

	$CO_2 + H_2$ liberated (μmoles)			
Time (hr.)	No substrate	Ribose	Ribose 5-phosphate	Inosine
1	1·29	1·29	4·02	2·16
2	1·92	2·30	8·36	4·82
3	2·23	4·03	9·38	7·72
4	2·61	6·88	10·05	10·52

Addition of phosphate (0·01–0·05 M) had no effect on the rate or fermentation of inosine by the combined sediment and supernatant fractions.

Effects of cetyltrimethylammonium bromide and lyzosyme on cell permeability

Cetavlon (cetyltrimethylammonium bromide) has been used previously (Eggleston, 1957) to increase the permeability of *E. coli* cells to amino acids and nucleotides. Repaske (1958) has recently described conditions for the partial lysis of *E. coli* cells by a mixture of tris-HCl buffer, ethylenediaminetetra-acetic acid (EDTA) and lysozyme (from egg white). Table 4 shows the effect of these reagents on the permeability, as indicated by the fermentability of inosine, ribose 5-phosphate and ribose. Ribose was not fermented in the presence of cetyltrimethylammonium bromide, but ribose 5-phosphate generated gas at a greater rate than did inosine; the fermentation of the latter substance was inhibited by Cetavlon by about 50 % during the first hour. In other experiments cells were pre-incubated for 50 min. with tris-HCl buffer (0·033 M, pH 8·2), EDTA (1·33 mM) and lysozyme (0·4 mg; The Armour Laboratories), in a volume of 3 ml., before addition of 1 ml. from the side bulb containing 0·4 ml. of 0·5 M-tris–potassium maleate buffer, pH 6·8, substrate and water. The final pH on mixing was 7·0. Some lysis of cells occurred (as shown by density measurements in a Beckman spectrophotometer at 660 mμ) and the cell suspension became slimy. Again free ribose was not fermented, and the rate of gas production from ribose 5-phosphate was faster than that from inosine during the first half hour. A feature of the cells treated with cetyltrimethylammonium bromide or lysozyme was that the liberation of gas from ribose 5-phosphate ceased when it had reached about half the volume of that obtained from inosine. In contrast, in cell-free suspension similar volumes of gas were evolved from the two components though at different rates (see Table 3).

Table 4. *Effects of cetyltrimethylammonium bromide and lysozyme on the fermentation of ribose, ribose 5-phosphate and inosine by intact cells*

Cells: 12·4 mg. dry wt. Other conditions were as given in Table 1. Cetyltrimethylammonium bromide (CTAB), where added, was mM in final concentration. Pretreatment with lysozyme was as described in the text. The data refer to μmoles of $CO_2 + H_2$ liberated after addition of substrate.

	$CO_2 + H_2$ liberated (μmoles)						
	Cells with no further treatment		Cells + CTAB		Cells preincubated with lysozyme		
Substrate							
Time (hr.)...	1	2	1	2	0·5	1	2
None	0·11	0·11	0·83	0·93	1·50	1·74	2·14
Ribose	0·26	0·29	1·18	1·29	1·67	1·99	2·64
Ribose 5-phosphate	0·20	0·36	4·33	4·35	3·48	5·45	5·54
Inosine	5·80	9·74	2·82	5·05	2·86	5·63	10·20

Various other substances were tested for their ability to facilitate the fermentability of ribose in whole cells. ATP, adenosine 5′-phosphate, inosine, hypoxanthine, glucose, inorganic phosphate, lysine and acid-hydrolysed casein had no effect.

Table 5. *Fermentation of ribose and ribose-containing compounds by intact cells grown in a ribose medium*

Whole cells: 8·2 mg. dry wt. Other conditions were as given in Table 1.

Substrate	$CO_2 + H_2$ liberated after addition of substrate (μmoles)		
Time (min.)...	30	60	90
None	0·71	0·85	1·32
ATP	1·72	2·46	3·62
Ribose 5-phosphate	3·35	5·80	5·95
Guanylic acid	4·47	5·85	6·39
Uridylic acid	2·68	5·22	6·92
Cytidylic acid	2·43	4·95	7·28
AMP-5′	3·26	6·96	7·86
Inosine	3·28	6·88	8·70
Ribose	3·60	8·45	9·11

Fermentation of ribose by cells grown in a medium containing ribose

When the glucose of the culture medium was replaced by ribose, cells were obtained which were capable of fermenting ribose readily (Table 5). Ribose reacted at a faster rate than ribose 5-phosphate or inosine and the total yield of gas from ribose 5-phosphate was again lower than that obtained in cell-free suspensions. As with cells grown on glucose, gas production from ATP was relatively slow. It was more rapid from adenylic acid, guanylic acid, uridylic acid, and cytidylic acid, inosine and ribose 5-phosphate, but all these substances reacted less rapidly than free ribose. Acetic acid and ethanol were identified among the end products. The proportions of the various products formed were not substantially different from those found with cells grown in the presence of glucose.

Induction of the fermentation of ribose in washed cells grown in the presence of glucose

Washed cells grown in the standard medium and incapable of fermenting free ribose acquired the capacity to ferment ribose if kept in ribose-

Table 6. *Development of the ability to ferment ribose in cells grown in a glucose medium*

Washed cells of *E. coli* 8571 (13–15 mg. dry wt.) were incubated in O_2 or N_2 at 25° for 5·5 hr. in pairs of Warburg vessels. The medium (2·8 or 3·0 ml.) contained KCl (0·01 M), $MgCl_2$ (3 mM), buffer, pH 6·8 (0·033 M), and the following concentrations of substrates (where added): ribose 0·013 M; ATP 1·6 mM; NH_4Cl 2 mM; HCl-hydrolysed casein (casein hydrol.) 1% (w/v); glucose 0·013 M; inosine 0·013 M. At the end of this 'pretreatment' period the gas space in the vessels was filled with N_2 and after further equilibration for 25 min. 0·2 ml. of 0·1 M-ribose was added from the side arm of one of each pair. Results are expressed as μmoles of gas evolved in 1 hr. from the time of adding the second amount of ribose.

	Conditions for the 5·5 hr. 'pretreatment' period			Gas evolved after the 'pretreatment' period (μmoles)	
Expt. no.	Gas phase	Buffer	Substrate	Without extra ribose	With extra ribose
1	O_2	Phosphate	Ribose	6·56	10·6
	O_2	Phosphate	Ribose + ATP	11·7	17·7
	O_2	Phosphate	Ribose + NH_4Cl	7·95	13·7
	O_2	Phosphate	Ribose + NH_4Cl + ATP	12·0	18·4
	O_2	Phosphate	Ribose + casein hydrol.	1·18	8·66
	O_2	Phosphate	Ribose + glucose	7·68	11·1
	N_2	Phosphate	Ribose + ATP	1·38	1·51
2	O_2	Tris–maleate	None	0	0
	O_2	Phosphate	None	0	0
	O_2	Tris–maleate	Ribose + ATP	5·36	12·3
	O_2	Phosphate	Ribose + ATP	9·06	17·0
	O_2	Phosphate	Inosine + ATP	0·71	2·59
	O_2	Phosphate	ATP	0	2·12
	O_2	Phosphate	ATP + glucose + casein hydrol.	9·82	11·3
3	O_2	Phosphate	Ribose	4·67	9·52
	O_2	Phosphate	Ribose + NH_4Cl (2 mM)	7·68	15·2
	O_2	Phosphate	Ribose + NH_4Cl (4 mM)	7·45	14·9
	N_2	Phosphate	Ribose + NH_4Cl (2 mM)	1·30	1·35
4	O_2	Phosphate	Ribose + NH_4Cl	8·30	14·7
	O_2	Phosphate	Ribose + NH_4Cl + chloramphenicol (10 μg./ml.)	—	1·05
	O_2	Phosphate	Ribose + NH_4Cl + chloramphenicol (20 μg./ml.)	—	0·54
	O_2	Phosphate	Ribose + NH_4Cl + chloramphenicol (40 μg./ml.)	—	0·11
	O_2	Phosphate	Ribose + NH_4Cl + chloramphenicol (60 μg./ml.)	—	0

containing media under the following conditions: the washed cells were shaken for 5·5 hr. at 25° in pairs of Warburg vessels in the medium described under Methods, containing KCl, $MgCl_2$ and a buffer of pH 6·8. In addition, ribose (0·4 ml.; 0·1 M) and other substances listed in Table 6 were present in a final volume of 3 ml. The gas space contained O_2. At the end of this 'pretreatment' period the O_2 was replaced by N_2 and after an equilibration period of 25 min. extra ribose (0·2 ml.; 0·1 M) was added from the side arm of one of each pair of vessels. An evolution of gas indicated that the cells had acquired the ability to ferment the added ribose. The control vessel to which no extra ribose was added measured the gas evolution from the residual ribose and from other substances, e.g. amino acids.

The presence of O_2 during the pretreatment was found to be essential for the development of the fermentability of ribose under the conditions stated, although the cells had been grown anaerobically and can acquire the ability to ferment added ribose if grown in a ribose-containing medium anaerobically under other conditions. The amount of cell material (dry wt.) did not increase during the incubation, indicating that the ability to ferment ribose was acquired without growth. As shown in Table 6 the addition of a source of nitrogen such as NH_4Cl, casein hydrolysate or ATP (which undergoes deamination and dephosphorylation) enhanced the development of the ability to ferment ribose. The effects of NH_4Cl and ATP were not additive but phosphate caused some small increases. When ribose was omitted from the pretreatment medium the cells remained unable to ferment added ribose and inosine did not replace ribose. Glucose caused an inhibition. The time curve of the rate of ribose fermentation after different periods of pretreatment shows that the maximum is reached in about 5–6 hr. (Fig. 1). At this stage the rate of ribose fermentation was about the same as in cells grown on ribose.

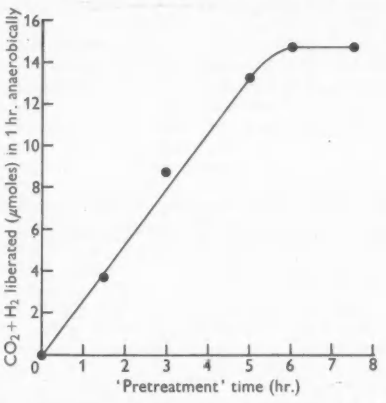

Fig. 1. Time curve of the rate of development of the fermentability of ribose in washed cells grown anaerobically in a glucose-containing medium. The cells were pretreated by shaking for various times at 25° with ribose and NH_4Cl in an atmosphere of O_2, as described in the text.

Chloramphenicol (chloromycetin) which is known to inhibit protein synthesis, prevents the development of the ability to ferment free ribose (Table 6, Expt. 4). Concentrations as low as 10 μg./ml. were sufficient to cause an inhibition of 93 %. The ribose-fermenting enzymes themselves were not inhibited by chloramphenicol concentrations of 60 μg./ml., as shown by the fact that inosine was fermented at the full rate in the presence of this concentration of chloramphenicol.

DISCUSSION

Ribose permease. The main findings of this investigation can be satisfactorily accounted for by the assumption that the penetration of ribose into the cells requires a 'permease' (Cohen & Monod, 1957) and that the formation of this permease depends on the presence of ribose in the medium. Different mechanisms must be responsible for the uptake of ribose and ribose 5-phosphate on the one hand and ribosides and ribotides on the other. Whether the penetration of ribotides and ribosides is also mediated by a permease or by simple diffusion remains to be investigated.

It is noteworthy that the fermentation of ribose 5-phosphate, but not of ribose, is very much enhanced by the addition of cetyltrimethylammonium bromide or lysozyme. The effects of these agents are likely to be due to a modification in the structure of the cell walls and they indicate that the barrier which prevents the fermentation of added ribose 5-phosphate may be overcome in at least two different ways, either by structural modifications or by a specific protein acting in an unknown fashion as a permease.

Strain differences. Although many strains of *E. coli* are known to possess enzymes required for degradation of nucleotides and nucleosides, the ability of washed cells to form gas from these compounds was found in one strain only of seven strains examined.

This strain also has other unusual properties not shared by the other six: (*a*) its ornithine decarboxylase is greatly accelerated by the addition of adenosine 5′-phosphate at the optimum pH (4·5), in contrast with the ornithine decarboxylase of other *E. coli* strains, which are accelerated by adenosine 5′-phosphate only at or near pH 7·0 (Eggleston, 1957); (*b*) it contains a 'factor' which

accelerates the phosphorolysis of citrulline by mammalian liver (Krebs, Eggleston & Knivett, 1955; Krebs *et al.* 1958).

Related observations. In a brief note Kopper (1956) reported that washed cells of *E. coli*, *Proteus vulgaris* and twelve other un-named species can reduce tetrazolium salts in the presence of various nucleosides but cannot do so on addition of free ribose or ribose phosphates. It is likely that this behaviour is related to the phenomena described in this paper.

SUMMARY

1. Washed intact cells of *Escherichia coli* strain N.C.I.B. 8571, grown semi-anaerobically in a glucose medium, are able to degrade anaerobically D-ribose added in the form of purine and pyrimidine nucleotides or nucleosides. The products formed include carbon dioxide, hydrogen, ethanol, acetic acid and succinic acid.

2. Free ribose and ribose 5-phosphate are not degraded by intact cells grown in a glucose medium, but are fermented by disintegrated cell material obtained by supersonic vibration.

3. Intact cells grown in a medium containing ribose instead of glucose rapidly ferment ribose and 5-phosphate, as well as the ribose moiety of purine and pyrimidine nucleotides.

4. Washed cells grown in a glucose medium acquire the ability to ferment ribose if they are incubated for a few hours in the presence of oxygen, ribose and a source of nitrogen.

5. The observations are in accord with the assumption that the penetration of ribose and ribose 5-phosphate into the cell is mediated by a specific permease.

6. Washed cells also acquire the ability to ferment ribose 5-phosphate (but not ribose) when treated with cetyltrimethylammonium bromide or lysozyme plus ethylenediaminetetra-acetic acid. These agents presumably cause structural modifications in the cell wall.

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Cholinesterase, Succinic Dehydrogenase, Nucleic Acids, Esterase and Glutathione Reductase in Sub-cellular Fractions from Rat Brain

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Differential centrifuging of homogenates of tissues in sucrose solution has indicated, particularly for liver, that enzymes are localized in particles of various sizes as well as in the soluble fraction. Fractions from liver homogenates have been extensively studied and various enzymes may now be used as markers so that the distribution of other enzymes may be compared with them (de Duve, Pressman, Gianetto, Wattiaux & Applemans, 1955).

Initially in this study our aim was to determine the distribution of cholinesterase in nervous tissue, but experiments showed that although a large proportion of the cholinesterase could be sedimented with the microsomal fraction there was also a

substantial amount in the mitochondrial fractions. This paper describes attempts to decide whether cholinesterase is entirely a microsomal enzyme by using the distribution of other enzymes for comparison. While this work was being carried out, Toschi (1959) gave a preliminary account of results of a study of cholinesterase in the microsomal fraction of brain.

METHODS

Special chemicals and reagents. The following chemicals were obtained from the sources indicated: oxidized glutathione (L. Light and Co. Ltd.); bovine serum albumin (fraction V, The Armour Laboratories); phenyl butyrate (British Drug Houses Ltd.); acetylcholine chloride (ACh) (Roche Products Ltd.); reduced triphosphopyridine nucleotide (TPNH) (Sigma Chemical Co., St Louis, Mo., U.S.A.); gelatin (bacteriological) (G. T. Gurr Ltd., London); ethylenediaminetetra-acetic acid (EDTA) (Hopkin and Williams Ltd.); eserine sulphate (Burroughs Wellcome Ltd.). Butyrylcholine perchlorate (BuCh) was prepared as described previously (Aldridge, 1953). Calf-thymus deoxyribonucleic acid (DNA) and yeast ribonucleic acid (RNA) were kindly supplied by Mr C. J. Threlfall, mipafox (*NN*-di*iso*propylphosphorodiamidic fluoride) by Dr G. S. Hartley (Fisons Pest Control Ltd.) and 62C47 [1:5-bis-(*p*-trimethylammoniumphenyl)pentan-3-one di-iodide] by Dr F. C. Copp (The Wellcome Foundation Ltd.).

Fractionation procedure. After decapitation of rats the brains were removed, rinsed in ice-cold 0·3 M-sucrose, roughdried and weighed. Two brains were homogenized in 15 ml. of ice-cold 0·3 M-sucrose by using a smooth glass tube and Perspex pestle with an all-round clearance of 0·005 in., as previously described (Aldridge, 1957). The homogenate was diluted to 25 ml. with 0·3 M-sucrose. The speed and time of centrifuging were varied for different fractions. The letters N, M_1 and M_2, P and S respectively are used to designate the nuclear, two mitochondrial, microsomal and supernatant fractions. The force and time of centrifuging is indicated by '*a*-fraction-*b*', where '*a*' is 10^{-3} *g*, 'fraction' is N, M_1, M_2, P or S, and '*b*' is time in min. As an example, $7M_1 15$ indicates a mitochondrial fraction sedimented from a suspension from which nuclei have previously been removed; the sedimentation force being 7000 *g* and the time 15 min. All separations were carried out at 0–2° with a Measuring and Scientific Equipment Ltd. refrigerated Angle 13 centrifuge or the Spinco model L centrifuge, and *g* values quoted refer to the bottom of the centrifuge tubes. With the exception of the microsomal fraction, all fractions were washed once and the wash fluid from each fraction was used to wash the next. The wash fluid and supernatant from the last mitochondrial fraction were combined and the microsomal fraction was then separated from this mixture. With the standard fractionation procedure the following mean recoveries of protein (five experiments, ±S.D.) were obtained: fraction 1 N 10, 15·6±1·8%; $7M_1 15$, 31·7±2·0%; $13M_2 15$, 8·9±0·4%; 145 P 60, 16·3±1·1%; S, 25·8±0·5%; total recovery 98·3±1·7%. The mean protein content of rat brain is 88·6±5·9 mg. of protein (as albumin)/g. wet wt. of brain.

Determination of cholinesterase. Tissue suspensions (0·5 ml.) in 0·3 M-sucrose were assayed at 37° manometrically in a medium containing (in final concn.) NaCl (0·13 M), $MgCl_2$ (35 mM), $NaHCO_3$ (31 mM), and equilibrated with CO_2+N_2 (5:95) (Aldridge, 1953). True cholinesterase and pseudocholinesterase were determined from the ratio of the rates of hydrolysis of acetylcholine (13·8 mM) and *n*-butyrylcholine (0·03 M) by using the substrate ratios for the enzymes of rat brain determined by Davison (1953). Cholinesterase activity bore a linear relation to the amount of fraction taken, and no increase in activity was found after disruption of particles by repeated freezing and thawing. In experiments with inhibitors, mipafox was incubated with the enzyme for 30 min. at 37° (Aldridge, 1953), whereas inhibition by 62C47 was determined without prior incubation (Bayliss & Todrick, 1956).

Determination of succinic dehydrogenase. The manometric method of Quastel & Wheatley (1938) was used. During other studies on liver mitochondria an iso-osmotic medium (Aldridge, 1957) supplemented with $NaHCO_3$ and ferricyanide was used, but it became clear that, to obtain maximum and consistent activity, particles must be damaged and a hypotonic medium was used. Reproducible results were obtained when a tissue suspension in water (1 ml.) was assayed at 37° in a medium containing (in final concn.) potassium phosphate (12·5 mM), $MgCl_2$ (3·5 mM), EDTA (0·6 mM), potassium succinate (22·5 mM), $NaHCO_3$ (25 mM) and $K_3Fe(CN)_6$ (12·5 mM). The phosphate, $MgCl_2$, EDTA and succinate were adjusted to pH 7·4 with KOH before the addition of $NaHCO_3$. Ferricyanide was in the side arm and the tissue in the centre compartment during gassing with CO_2+N_2 (5:95). After gassing for 5 min. and equilibration for 5 min., ferricyanide was tipped in from the side arm and readings were taken at 15, 20, 25, 30, and 35 min. Enzyme activity was calculated from the best straight line through these readings (Aldridge, Berry & Davies, 1949).

Determination of glutathione reductase. Tissue suspensions (0·4 ml.) in water were twice frozen and thawed, and assayed in a medium (2·5 ml.) containing potassium phosphate (pH 7·4; 0·05 M), EDTA (0·33 mM), oxidized glutathione (0·3 mM) and TPNH (0·083 mM) (McIlwain & Tresize, 1957). The rate of change of extinction at 340 mμ at room temperature (22°) was determined graphically from readings in 1 cm. cells with a Unicam SP. 500 spectrophotometer. The rate of oxidation of TPNH in the presence and absence of glutathione was determined, the difference being glutathione reductase activity. Oxidation of TPNH in the absence of glutathione was significant for the mitochondrial fraction ($7M_1 15$), being 30–40% of the glutathione reductase activity.

Determination of esterase activity. Tissue suspensions (0·5 ml.) in 0·3 M-sucrose were assayed at 37° in a medium containing (in final concn.) $NaHCO_3$ (13 mM) and gelatin (1%, w/v) (Aldridge, 1954). Phenyl butyrate was added as an emulsion (70 μl./flask) in the $NaHCO_3$–gelatin medium. Tissue suspensions were tipped in from the side arm after gassing and equilibration with CO_2+N_2 (5:95). With this procedure the output of CO_2 was not quite linear with time. Reduction of the substrate concentration to 23 μl./flask and the addition of bovine serum albumin (1·5%, w/v) and $MgCl_2$ (35 mM) rectified this but made no difference to the relative activities of different fractions from brain or to homogenate activity.

Determination of nucleic acids. The method of Logan, Mannell & Rossiter (1952) was used except that 8% (w/v) perchloric acid was used instead of 10% (w/v)

trichloroacetic acid for the original precipitation, and 3% perchloric acid instead of 5% trichloroacetic acid for extraction of nucleic acid: final readings were at 266 mμ. All materials were kept ice-cold throughout procedures before the final acid extraction. Extraction of albumin gave low extinctions at 266 mμ, and the sum of extinctions obtained on separate extraction of albumin and RNA equalled that obtained from mixtures of these materials. DNA used as standard was a preparation from calf thymus (Gulland, Jordan & Threlfall, 1947), and the RNA was a commercial preparation from yeast purified by repeated solution in alkali and reprecipitation with acid. At 266 mμ both these preparations had a molar extinction coefficient (based on P content) of 9930.

Determination of protein. Protein was precipitated with 8% (w/v) perchloric acid. The centrifuged pellet was rinsed with 5 ml. of ethanol–ether (55:45) and resuspended in 10 ml. of ethanol–ether at room temperature for 30 min. After centrifuging, the defatted pellet was dissolved in 1 ml. of 3% (w/v) NaOH with warming and the proteins determined by the method of Robinson & Hogden (1940) as modified by Aldridge (1957). Opalescence in the final coloured solution was avoided by this removal of lipid. The results have been expressed as milligrams of albumin.

RESULTS

Our early studies on cholinesterase showed that it was diffusely distributed among the fractions we were preparing. We therefore switched to the study of some probable mitochondrial and nuclear markers to assess the extent of mutual contamination of fractions. These results are reported first, and on them we based our modified centrifuging conditions for further cholinesterase study. Finally we studied distribution of esterase and glutathione reductase for comparison with cholinesterase.

Succinic dehydrogenase. Previously (Aldridge, 1957) it was shown that a homogenizer with an all-round clearance of 0·01 in., although suitable for the isolation of liver mitochondria, was unsuitable for brain since a large proportion of the succinic dehydrogenase sedimented at 1000 *g* and was presumably associated with unbroken cells. Experiments were therefore carried out to check that the homogenizer with an all-round clearance of 0·005 in. was breaking the majority of cells. Further work was done to select centrifugal speeds and time such that succinic dehydrogenase could be obtained in two mitochondrial fractions. This was important, since in our initial experiments some cholinesterase sedimented in the mitochondrial fraction and we wished to compare the specific activities of succinic dehydrogenase and cholinesterase of two mitochondrial fractions. After sedimentation at 1000 *g* for 10 min., only 10% of the succinic dehydrogenase was found in the nuclear fraction (Table 1). Decrease of the homogenizer clearance from 0·005 to 0·002 in. did not appreciably lower the succinic dehydrogenase content of the nuclear fraction (Table 2). Most of the succinic dehydrogenase had sedimented after 13 000 *g* for 15 min. As our standard procedure therefore we have used 1000 *g* for 10 min. for the nuclear fraction, 7000 *g* and 13 000 *g* for 15 min. to give two successive mitochondrial fractions, and a final 145 000 *g* for 1 hr. for the microsomal fraction. No attempt was made to subfractionate the microsomal fraction. The distribution of succinic dehydrogenase with this technique is shown in Table 2.

Nucleic acids. DNA is accepted as a suitable marker for nuclei (Davidson, 1957). In view of the large range of nuclear size in brain (Ham, 1953) it was desirable to have a quantitative measure of nuclear contamination of other fractions. With the

Table 1. *Sedimentation of succinic dehydrogenase from homogenates of rat brain by different centrifugal speeds and times*

For experimental details and meaning of fraction designations, see Methods section, under Fractionation procedure. In these preliminary experiments the washings from the nuclear fraction were not used to wash the mitochondrial fraction.

	Percentage of total homogenate activity	
Fraction	Sedimented	Not sedimented
0·7N10	10·4	—
1·0N10	11·7, 9·6, 7·1	—
1·3N10	16·1	—
$4M_1$15	—	18·4
$5M_1$15	—	15·2, 11·5
$7M_1$15	—	9·1
$9M_1$15	—	6·5
$10M_1$15	—	3·5, 5·9
$13M_1$15	—	3·3, 5·4

Table 2. *Comparative distribution of succinic dehydrogenase and cholinesterase in fractions from homogenates of rat brain*

Two homogenates (homogenizer, all-round clearance 0·005 in.) of two rat brains each were combined and then divided in two. One (Exp. 2) was rehomogenized (homogenizer, clearance 0·002 in.). SD, succinic dehydrogenase; ChE, cholinesterase.

	Recovery of homogenate activity in fraction (%)			
	Expt. 1		Expt. 2	
Fraction	SD	ChE	SD	ChE
1N10	7·7	8·7	6·1	7·5
$7M_1$15	70·4	26·8	65·7	24·4
$13M_2$15	13·6	15·3	11·5	14·1
145P60	5·8	37·2	4·6	45·3
S	2·4	10·3	0·2	12·1
Total	99·9	98·3	88·1	93·4
Homogenate activity (μl. of CO_2/g. of brain/hr.)	13 800	12 200	15 600	11 200

Table 3. *Distribution of nucleic acids in fractions from homogenates of rat brain*

Values are the mean of results from two experiments (except for the supernatant, which is one experiment only). The values for specific activity (μg. of nucleic acid P/mg. of protein) were calculated with the protein distribution given in the Methods section. The mean nucleic acid content of the brains used was: total nucleic acid, 225; DNA, 135; RNA, 90 μg. of nucleic acid P/g. wet wt.

Fraction	Total nucleic acid (%)	DNA (%)	DNA (μg. of P/mg. of protein)	RNA (%)	RNA (μg. of P/mg. of protein)
1N10	62·9	77·2	7·6	41·6	2·8
$7M_1$15	9·6	8·4	0·4	11·5	0·4
$13M_2$15	3·5	1·7*	0·3*	6·4	0·7
145P60	18·2	1·8*	0·2*	42·2	2·7
S	3·9	2·7*	0·2*	5·9*	0·2*
Homogenate	100	100	1·5	100	1·0
Total of fractions	102·3	90·8	—	106·4	—

* Results were based on extinctions of <0·10 and therefore of low accuracy.

Table 4. *Distribution of cholinesterase in fractions from homogenates of rat brain*

Results are given as mean ±S.D. of seven experiments for percentage total cholinesterase (ChE), and of four experiments for units/mg. of protein. The values for true cholinesterase and pseudocholinesterase were obtained from a single experiment. Substrates are as indicated and interference corrections are described in the Methods section. 1 unit of ChE activity is 1 μl. of CO_2/hr. Total ChE activity (ACh) of brain was 11 700±1200, true ChE (ACh) 11 100 and pseudo ChE (BuCh) 1530 units/g. wet wt.

Fraction	Total ChE (ACh) (%)	Total ChE (ACh) (Units/mg. of protein)	True ChE (ACh) (%)	True ChE (ACh) (Units/mg. of protein)	Pseudo ChE (BuCh) (%)	Pseudo ChE (BuCh) (Units/mg. of protein)
1N10	9·1±0·8	78±11	6	55	36	47
$7M_1$15	26·9±1·6	113± 9	27	116	19	11
$13M_2$15	14·0±1·2	216±18	16	226	7	14
145P60	38·2±4·3	316±28	38	305	24	26
S	13·6±2·3	70±13	9	44	27	19
Homogenate	100	136± 9	100	133	100	18
Total of fractions	101·5±2·9	—	96	—	113	—

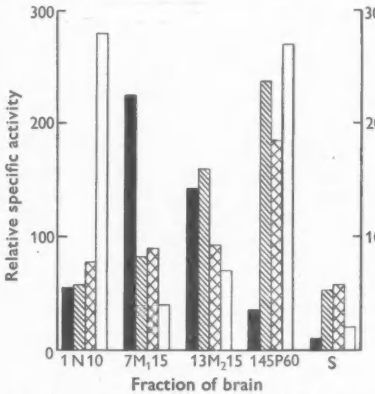

Fig. 1. Histogram of the specific activities of enzymes and RNA in sub-cellular fractions of rat brain. ■, Succinic dehydrogenase; ▨, cholinesterase; ▩, esterase; □, RNA. Specific activities of homogenate were taken as 100 for comparison, and (per mg. of protein) were: succinic dehydrogenase, 165 μl. of CO_2/hr.; cholinesterase, 136 μl. of CO_2/hr.; esterase, 220 μl. of CO_2/hr.; RNA, 1·0 μg. (P).

standard fractionation procedure, DNA is predominantly located in the nuclear fraction with a small contamination of the first mitochondrial fraction but with insignificant amounts in other fractions (Table 3). RNA is located both in the nuclear and microsomal fraction (Table 3 and Fig. 1). These distributions of nucleic acids are very similar to those obtained by Brody & Bain (1952).

Cholinesterases. The initial experiments showed that most of the cholinesterase was not sedimented in the nuclear or mitochondrial fractions. Upon high-speed centrifuging 35–40% of the cholinesterase sedimented in the microsomal fraction (Table 4).

There was, however, 27% of the cholinesterase activity in the heavy mitochondrial fraction ($7M_1$15, 105 000 *g*-min.) and 14% in the light mitochondria ($13M_2$15, 195 000 *g*-min.). This is considerably more than is obtained by microsomal contamination of liver mitochondria. For instance, de Duve *et al.* (1955) recovered 2·7% of the liver glucose 6-phosphatase in their heavy (33 000 *g*-min.) and 7% in their light mitochondrial fractions (250 000 *g*-min.). Since brain has a very low glucose

6-phosphatase activity (Dixon & Webb, 1958) this enzyme cannot be used there as a microsomal marker. The specific activities of cholinesterase in brain fractions (Table 4 and Fig. 1) show that the distribution is consistent with a large microsomal contamination of the mitochondrial fractions. RNA is also concentrated in the microsomes but the proportion found in the mitochondrial fractions is much less than with cholinesterase (Table 3 and Fig. 1).

Use of acetylcholine and *n*-butyrylcholine to determine true cholinesterase and pseudocholinesterase in the various fractions reveals a marked difference in the distribution of the two enzymes (Table 4). The specific activity of pseudocholinesterase is highest in the nuclear fraction, though it is present in the other fractions. The pseudocholinesterase of the nuclear fraction was inhibited by 6 μM-mipafox and unaffected by 0·1 mM-62C47, in agreement with results on whole rat brain obtained by Davison (1953) and Bayliss & Todrick (1956), respectively. The ratio of rates of hydrolysis of substrates by this enzyme (butyrylcholine:acetylcholine) was found to be 141:100, in excellent agreement with Davison (1953). Since 90% of the cholinesterase activity is due to true cholinesterase the distribution of this enzyme is not appreciably different from that of the total cholinesterase activity.

Esterase. With phenyl butyrate as a substrate the distribution of esterase in rat-brain fractions was examined. The distribution is fairly similar to that of cholinesterase, the only difference being that a lower percentage of esterase is always found in the microsomal fraction (Table 5 and Fig. 1).

Glutathione reductase. In view of the unequal distribution of RNA, cholinesterase and esterase, other enzymes were examined. McIlwain & Tresize (1957) demonstrated the high rate of oxidation of reduced TPNH by oxidized glutathione in cerebral tissues, and the results in Table 6 show that the glutathione reductase was present mainly in the mitochondrial fraction and in the supernatant. Washing with buffered electrolyte solution (pH about 7·5) containing glycylglycine, potassium chloride and magnesium chloride did not remove the activity from the mitochondrial fraction. These results are different from those for liver: Rall & Lehninger (1952) found that after centrifuging liver homogenate at 20 000 *g* for 20 min., 90% of the glutathione reductase was present in the supernatant fraction.

Table 5. *Distribution of esterase in fractions from homogenates of rat brain*

1 unit of activity is 1 μl. of CO_2/hr. Results are the mean ±S.D. of three experiments. The mean activity of brain is 20 000±1750 units/g. wet wt. In one experiment it was shown that the esterase in each of the fractions was not inhibited by 20 μM-eserine.

Fraction	Activity (%)	Activity (Units/mg. of protein)
1N10	13·1±1·1	171±28
7$M_1$15	29·1±0·3	199±32
13$M_2$15	7·7±0·5	202±25
145P60	26·8±3·5	406±55
S	14·5±1·1	127±23
Homogenate	100	220±28
Total of fractions	91·2±4·1	—

Table 6. *Distribution of glutathione reductase in fractions from homogenates of rat brain*

1 unit of glutathione reductase activity is a change in extinction at 340 mμ of 1·0/min., measured in 1 cm. cells at room temperature (22°), the final volume being 2·5 ml. Activity of brain is 1·82 units/g. wet wt.

Fraction	Activity (%)	Activity (Units/100 mg. of protein)
1N10	11·9	1·56
7$M_1$15	28·3	1·84
13$M_2$15	7·1	1·64
145P60	9·5	1·20
S	43·9	3·50
Homogenate	100	2·05
Total of fractions	100·7	—

DISCUSSION

It is necessary to establish that we have chosen suitable markers for mitochondria and nuclei.

Although most of the evidence for the use of succinic dehydrogenase as a marker enzyme for mitochondria has been obtained with liver (Schneider & Hogeboom, 1956; de Duve *et al.* 1955; Kuff, Hogeboom & Dalton, 1956), mitochondria from other tissues such as heart, skeletal muscle and kidney also contain succinic dehydrogenase. There is no reason from other work (Abood, Gerard, Banks & Tschirgi, 1952; Brody & Bain, 1952) or from the present study to believe that brain mitochondria differ in this respect. On the basis that succinic dehydrogenase is exclusively located in the mitochondria, the nuclear fraction contains 7–10% of either unbroken cells or mitochondria.

The distribution of DNA is accepted as a marker for nuclei (Davidson, 1957). The distribution of DNA obtained in this study is essentially the same as that found by Abood *et al.* (1952) and Brody & Bain (1952), and indicates a contamination of our first mitochondrial fraction (7 $M_1$15) with 10–15% of the nuclear DNA. This is confirmed by histological examination.

Having chosen our fractionation procedure, it

became obvious that cholinesterase was present in highest quantity and specific activity in the microsomal fraction but was also present to a considerable extent in the mitochondrial fraction. The specific activity increased from fraction 7 $M_1$15 through 13 $M_2$15 and was maximal in the microsomal fraction (Fig. 1).

When we compare the distribution of cholinesterase with that of esterase and RNA, both of which we find in the microsomal fraction to a considerable extent, there is no clear correlation. We showed that esterase is distinct from cholinesterase in that the former activity in whole homogenate and in microsomes was not affected by 20 μM-eserine, whereas the cholinesterase of these fractions was about 95 % inhibited. The distribution of esterase is similar to that of cholinesterase, but in our opinion is sufficiently different not to be considered identical (Fig. 1): whether this is a real difference in distribution or a technical artifact must remain in doubt because the recovery of esterase from the fractions was persistently lower (91 %) than that of cholinesterase (101 %). The possibility must also be considered that the hydrolysis of phenyl butyrate is due to more than one enzyme (Myers, 1956). The distribution of RNA is bimodal, highest concentrations being found in the nuclear and microsomal fractions (Table 3; cf. Abood *et al.* 1952; Brody & Bain, 1952). From the contamination data deduced from DNA-distribution studies, it may be assumed that some RNA found in the heavy mitochondrial fraction originated in the nuclei. Even if this were not so, the percentage of 'microsomal' RNA in the mitochondrial fractions is considerably less than that of cholinesterase and the distributions are clearly different (Fig. 1).

In work carried out concurrently with ours it has been shown that microsomal fractions from rat brain (Hanzon & Toschi, 1959; Toschi, 1959) contain both cholinesterase and RNA. The cholinesterase may be partially separated from RNA by centrifuging and by various treatments such as with deoxycholate, some detergents or ribonuclease. Toschi has correlated these findings with electron micrographs of the fractions and considers that cholinesterase is concentrated in a 'heavy' fraction whereas RNA is associated with electron-dense granules which are concentrated in a 'light' fraction. Frontali & Toschi (1958) have reported a similar association of cholinesterase with membranous structures present in sub-cellular fractions from electric tissue of *Torpedo*.

Glutathione reductase was examined in the hope of finding a similarity to cholinesterase distribution, but its two peaks of activity appear in the mitochondrial and supernatant fractions with little in the microsomes (Table 6). The presence of some enzyme in a particulate fraction probably explains the activation which was reported to be obtained by treating brain homogenates with hypotonic solutions (McIlwain & Tresize, 1957).

A comparison of the distribution of enzymes in liver with those in brain reflects the different complexity of organization in these two tissues. Liver contains a preponderant mass of cells of similar size, whereas brain contains a great variety from large neurons down to microglia, and it is not therefore surprising that, with brain, less clear-cut conclusions can be reached. It may be that the present homogenization and centrifuging procedures will never be suited to separation of brain-cell components, and the failure to obtain brain mitochondria capable of sustained oxidative phosphorylation (Aldridge, 1957) may also reflect the inadequacy of this technique. However, in liver, RNA distribution is bimodal (Hogeboom & Schneider, 1955), as in brain, and there is some indication that microsomal diphosphopyridine nucleotide–cytochrome *c* reductase may be separated into different ranges of particle size (Kuff *et al.* 1956). Esterase has also been found mainly in the microsomal fraction from liver (Omachi, Barnum & Glick, 1948; Novikoff, Podber, Ryan & Noe, 1953). The small amount of cholinesterase present in rat liver was also mainly present in the microsomal fraction (Underhay, Holt, Beaufay & de Duve, 1956). Similar distributions are reported for glucose 6-phosphatase, esterase and cholinesterase (Underhay *et al.* 1956), about 60–70 % being found in rat-liver microsomes, and the distribution of RNA has been reported to be similar to that of esterase (Novikoff *et al.* 1953). It is obviously undecided whether RNA and microsomal enzymes are located in the cell on different structures, or whether the differences in distribution are artifacts produced by the homogenization procedure.

The finding of so large a proportion as 36 % of total pseudocholinesterase in the nuclear fraction is worthy of comment. This is a higher proportion than can be explained by the presence of unbroken cells. As previously pointed out, the maximum amount of unbroken cells must be 7–10 % of the total, assuming the general distribution of succinic dehydrogenase throughout the cellular matter of brain (Burgen & Chipman, 1951; Shimizu & Morikawa, 1957). Some evidence has been given on the other hand that pseudocholinesterase is present mainly in the glial cells and especially fibrous astrocytes (Koelle, 1950, 1954; Cavanagh, Thompson & Webster, 1954). It is therefore possible that many of the unbroken cells are glial cells; histological examination has been made but no definite conclusions were drawn. The original suggestion from histochemical work (Koelle, 1950) that the nuclei or the nuclear membrane in glial cells contain pseudocholinesterase in high concentrations has not

been substantiated (Koelle, 1954; Ravin, Zacks & Seligman, 1953; Crevier & Bélanger, 1955). The possible diffusion of pseudocholinesterase from the cytoplasm to the nuclei after sectioning (Koelle, 1951) indicates that much caution must be used before assuming that the nuclei contain pseudocholinesterase. However, other workers have found cholinesterase in the nuclear fraction from liver (Underhay *et al.* 1956), and nuclei isolated from rat liver by the method of Dounce (1950) contained one-quarter of the pseudocholinesterase (Koelle, 1951).

A snare inherent in all fractionation studies is the possibility of the absorption of soluble material on to particulate matter as happens with arginase (Rosenthal, Gottlieb, Gorry & Vars, 1956). In that case it was shown that the enzyme was readily freed from the nuclear and microsomal fractions by simple washing with iso-osmotic electrolyte solution. We therefore washed fractions of brain with buffered iso-osmotic solutions containing potassium chloride and magnesium chloride: pseudocholinesterase was not removed from nuclear or microsomal fractions, nor were true cholinesterase and glutathione reductase removed from mitochondrial fractions.

SUMMARY

1. Homogenates of rat brain in 0·3 M-sucrose have been separated by centrifuging into a nuclear fraction, two mitochondrial fractions, a microsomal and a supernatant fraction.

2. Succinic dehydrogenase and deoxyribonucleic acid have been used to assess the contamination of fractions by mitochondria and nuclei, respectively.

3. Of the true cholinesterase, 38 % is found in the microsomal fraction with a specific activity 2·6 times that of the homogenate. It was not possible to obtain other fractions free from cholinesterase.

4. The distribution of ribonucleic acid and cholinesterase is different.

5. Esterase (substrate, phenyl butyrate) has a distribution similar to but not identical with that of cholinesterase.

6. The nuclear fraction contains 36 % of the pseudocholinesterase, the microsomal fraction 24 % and the supernatant fraction 27 %.

7. Of the glutathione reductase, 35 % is present in the mitochondrial and 44 % in the supernatant fractions.

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The First Glycine Metabolic Pool in Man

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The number of reported investigations which have been designed to determine directly with the help of isotopically labelled intermediates whether a given precursor–product relationship operates in man *in vivo* are still relatively few. In connexion with our studies on the conversion of glycine into oxalate in subjects with primary hyperoxaluria (Scowen, Crawhall & Watts, 1958), we required to know the extent of the dilution which an oral dose of [^{13}C]glycine would undergo in the human subject. We thus calculated the size and turnover rate of the pool of glycine with which such a dose of glycine mixes immediately after absorption and distribution, and have called this pool the 'first glycine metabolic pool', a term previously used by Arnstein & Neuberger (1951). The location of this metabolic pool cannot be defined in anatomical terms, but the uncombined visceral glycine together with the glycine which is dissolved in the plasma and interstitial fluid presumably constitute a large part of it. The free urinary glycine is separated directly and continuously from the plasma by glomerular filtration. After the administration of isotopically labelled glycine, the isotope content of glycine isolated from urine would be expected to be the same as the isotope content of the plasma glycine at the moment of filtration, and we suggest that it may therefore be used as a measure of the isotope content of the glycine in the first glycine metabolic pool. The glycine moiety of the urinary hippuric acid that is excreted after oral doses of sodium benzoate which are sufficiently large to increase the urinary excretion of hippurate has been used as a sample of the glycine metabolic pool (Gray & Neuberger, 1950; Berlin, Hewitt & Lotz, 1954). This can be criticized on the grounds that the administration of these loading doses of sodium benzoate may cause the deviation of glycine from other metabolic pathways to participate in hippurate synthesis (Arnstein & Neuberger, 1951), or promote the synthesis of glycine from non-nitrogenous precursors (Gray & Neuberger, 1950). Moreover, since the liver is the main site of hippurate synthesis, dietary glycine reaching the liver via the portal vein might take part in this reaction before it had mixed uniformly in the glycine metabolic pool. We have studied this problem in man by comparing the isotope contents of the urinary hippuric acid and the first glycine metabolic pool during a period of repetitive feeding with, and after a single dose of, isotopically labelled glycine.

Stable isotopes were used in the present work as in the subsequent clinical investigations to eliminate any slight risk of radiation injury. Some of the results presented here have been the subject of preliminary communications (Crawhall & Watts, 1958*a*, *b*).

METHODS

Analyses were by Weiler and Strauss, Oxford. Melting points are uncorrected.

Synthesis of [1-^{13}C]*glycine and* [^{15}N]*glycine*

[1-^{13}C]Glycine had to be synthesized on a much larger scale than in previously described isotopic syntheses (cf. Arnstein & Bentley, 1950), and it was therefore necessary to design a new apparatus for the preparation of the first intermediate, [1-^{13}C]acetic acid. The direct amination of bromoacetic acid with NH_3 and $(NH_4)_2CO_3$ proved unsatisfactory and was abandoned, as we were unable to separate glycine from iminodiacetic acid on our scale of work (cf. Chase & Downes, 1953), the latter impurity being present to the extent of about 10% of the glycine yield in our preliminary experiments.

[1-^{13}C]*Acetic acid from* $Ba^{13}CO_3$. The reaction was performed in the carbonation apparatus (Fig. 1), which was not assembled until the Grignard reagent had been prepared in the triple-necked 3 l. flask (*A*). Ether-washed Mg-turnings (16 g., 0·6 mole) were covered with diethyl ether (1 l. previously dried over Na wire) and methyl iodide (41·4 ml., 0·6 mole) was added dropwise at such a speed as to keep the ether boiling gently under reflux. When the reaction appeared complete, the reagent was heated under reflux for 1 hr. The flask was then cooled and incorporated into the carbonation apparatus. The gas-inlet tube (Fig. 1) was of wide bore (8 mm. internal diam. proved satisfactory), as the Grignard complex which separates out as the carbonation proceeds tends to occlude it. The Hg-sealed paddle stirrer was operated by a powerful low-speed motor. The Dewar-type reflux condenser (*B*) was filled with a solid CO_2–ethanol cooling mixture. Spiral trap (*C*) was cooled in liquid O_2 to trap any unreacted CO_2; trap (*D*) was cooled in solid CO_2–ethanol and served to prevent the diffusion of water vapour into the apparatus. The bubbler (*E*) contained phosphoric acid (84%, w/v) and indicated the rate of flow of gas through the apparatus. Flask (*F*) contained saturated $Ba(OH)_2$ to collect any unreacted $^{13}CO_2$ not trapped in (*C*). This $Ba(OH)_2$ was only faintly opalescent at the end of the reaction. $Ba^{13}CO_3$ (66·1 g., 0·3 mole; 60·4 atoms % excess

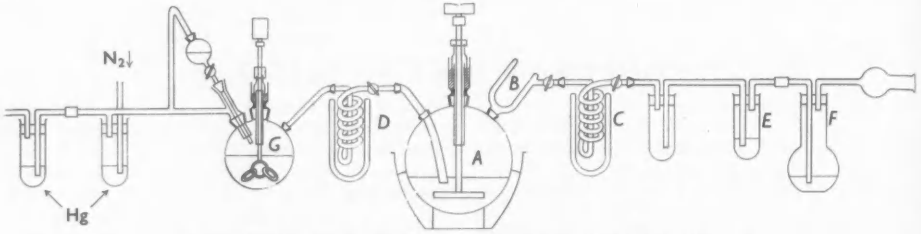

Fig. 1. Carbonation apparatus. For explanation see Methods section.

of ^{13}C) was placed in the triple-necked flask (*G*). This was fitted with a Hg-sealed paddle stirrer, an N_2-inlet tube and a dropping funnel, from which phosphoric acid (100 ml.; 84%, w/v) was added dropwise. Flask (*G*) was connected with flask (*A*) through the spiral trap (*D*), which was cooled in solid CO_2–ethanol to retain any water liberated from the phosphoric acid. Before commencing the liberation of CO_2, flask (*A*) was cooled to $-10°$, in solid CO_2–ethanol and maintained at this temperature throughout the course of the reaction. Nitrogen was passed through the apparatus at about 30 ml./min. The phosphoric acid was added to the $Ba^{13}CO_3$ over the course of about 1½ hr.; initially the stirrer in flask (*G*) had to be turned by hand but as the reaction proceeded the contents of the flask became more fluid and mechanical stirring was possible. When the addition of phosphoric acid was complete flask (*G*) was heated with a naked flame to ensure that all the $^{13}CO_2$ had been evolved. The Grignard reaction mixture was then stirred for a further 0·5 hr. The liquid O_2 trap (*C*) was quickly placed in the position of the solid CO_2–ethanol trap (*D*) and the system of traps beyond flask (*A*) was brought together again. Nitrogen was passed through to sweep any unreacted $^{13}CO_2$ back into the reaction mixture, and stirring was continued for a further ½ hr., after which the inlet tube of flask (*A*) was removed and replaced by a dropping funnel. Sulphuric acid (350 ml.; 16%, w/v) was added to the Grignard complex, the temperature of the reaction flask being maintained below 35°. Much gas was liberated (probably mainly methane) but no CO_2 appeared to be collected in the $Ba(OH)_2$ trap. When this reaction appeared to be complete Ag_2SO_4 (150 g.) was added to remove I^- ions and the reaction flask (*A*) was transferred to a steam bath and warmed gently under reflux with mechanical stirring until no more I_2 was visible in the ether layer. Insoluble material (AgI) was removed by centrifuging and filtration, and the residue was washed with 0·1 N-H_2SO_4 (2 × 100 ml.) and water (100 ml.). The combined aqueous phase and washings were distilled to dryness, water (300 ml.) was added to the non-volatile residue ($MgSO_4$) and steam-distillation was performed until the distillate showed a neutral reaction. The combined distillates were brought to pH 10·0 by the addition of approx. 5 N-NaOH and the water was removed by distillation. Sulphuric acid (50%, v/v) was added to the concentrated residue to give pH 1·0 and the solution was extracted for 24 hr. with ether (200 ml.) in a continuous-extraction apparatus. The ether was removed by slow distillation from a water bath (45°). The residue from this procedure was transferred to a Vigreaux flask, the conventional fractionating column of which had been replaced by a column (15 × 1 cm.) packed with Fenske helices, and the distillation was continued from an oil bath. The fractions collected between 100° and 118° had a combined weight of 17·34 g. and contained 16·34 g. (0·27 mole) of acetic acid (determined by titrating weighed 10 μl. portions of each fraction) with 1 g. of water (by difference). Yield, 90% based on $Ba^{13}CO_3$.

[1-^{13}C]*Bromoacetic acid.* [1-^{13}C]Acetic acid (15·8 g., 0·26 mole; 60·4 atoms % excess of ^{13}C) in conjunction with water (1 g.; 0·056 mole) as impurity was mixed with acetyl chloride (4·8 g.; 0·061 mole) in order to remove the water. Red phosphorus (0·3 g.) was then added and the mixture heated in a 100 ml. round-bottomed flask fitted with a dropping funnel and reflux condenser in an oil bath at 80°. Bromine (62·2 g., 0·78 mole; dried under H_2SO_4) was added dropwise during 2 hr. so that a gentle stream of HBr was evolved and the temperature retained at 80–100°. This temperature was maintained for a further 4 hr. The reaction mixture solidified on cooling. It was then distilled from an oil bath at reduced pressure (14 mm. Hg). A fraction (2 g.) was obtained at 60°. The main crop distilled at 110° (40·3 g., 0·29 mole; 90% based on total 'acetyl'). Most of this crystallized on cooling though some remained liquid, which may have been bromoacetyl bromide.

Ethyl bromo-[1-^{13}C]*acetate.* Bromo-[1-^{13}C]acetic acid (40·3 g.) was melted and transferred to a 250 ml. round-bottomed flask. A mixture of benzene and ethanol (80 ml.; 5:3) was added and the mixture heated under reflux on a steam bath with a Dean and Stark azeotropic distillation head. Water was separated by this apparatus and removed. After 2 hr. no more water was being formed, so extra benzene–ethanol mixture (50 ml.) was added and refluxing continued for a further 3 hr. The mixture was then poured into saturated $NaHCO_3$ soln. (100 ml.) and ether (100 ml.). The ether phase was separated and the aqueous phase again extracted with ether (2 × 100 ml.). Ether extracts were combined and dried over anhydrous Na_2SO_4. Sodium sulphate was removed by filtration and the ether solution distilled through a Vigreaux-type lagged fractionating column (1 in. × 11 in.) until the temperature rose to 56°. The residue was transferred to a 100 ml. Vigreaux flask and heated by an oil bath. The main fraction distilling between 150 and 153° was collected (42·5 g., 0·26 mole; 80% based on [^{13}C]acetic acid).

[1-^{13}C]*Glycine.* Ethyl bromo-[1-^{13}C]acetate (42·6 g.; 0·255 mole) was added to potassium phthalimide (49·3 g.; 0·266 mole) and heated by an oil bath at 115–120° under reflux for 4 hr. with occasional stirring. The mixture was never more than semi-fluid but the use of dimethylform-

amide as a solvent did not improve the yield. The residue, whilst still warm, was added to conc. HCl–acetic acid–water (1 l.; 1:1:1) and heated under reflux for 3 hr. Some of the solvent (800 ml.) was then removed by distillation. Conc. HCl–acetic acid (1 l.; 1:1) was then added and heating under reflux continued for 24 hr. The acid was again removed by distillation until phthalic acid began to crystallize. The mixture was cooled and the phthalic acid removed by filtration. The solid residue was carefully washed with water (500 ml.). The filtrate and washings were combined and the rest of the solvent was removed by distillation at reduced pressure from a water bath. The residue was dried *in vacuo* over P_2O_5 and NaOH flakes. The residue was then suspended with stirring in ethanol (300 ml.) for 0·5 hr. The insoluble residue was removed by filtration and washed carefully with ethanol (2 × 150 ml.). Water (100 ml.) was added to the combined filtrates to prevent esterification of the amino acid and the solvent removed by distillation *in vacuo*. The residue was dried and then dissolved in water (2 × 25 ml.). There was an insoluble brown residue which was removed by filtration. The amino acid hydrochloride solution was then passed through a column of Amberlite 1R-45 (2 × 60 cm.) in the OH form and eluted with water. The first five fractions (50 ml.) were combined and the water was removed by distillation. The residue was dried *in vacuo*. Yield 16·2 g. (0·216 mole; 85% yield based on ethyl bromoacetate; 59% yield based on $Ba^{13}CO_3$). It gave a single spot corresponding to glycine when tested chromatographically (Found: C, 32; H, 6·5; N, 18·1; $C_2H_5O_2N$ requires C, 32; H, 6·7; N, 18·7%). Attempts had been made in preliminary experiments to isolate glycine from the ethanol solution of its hydrochloride by precipitation with pyridine. This gave rise to a compound, m.p. 182°, which was probably diglycine hydrochloride (Bentley, 1951) (Found: C, 25·6; H, 5·7; N, 13·8. Calc. for $C_4H_{11}O_4NCl$: C, 25·7; H, 5·9; N, 14·9%).

[^{15}N]*Glycine*. $^{15}NH_4NO_3$ (4·2 m-moles) was converted into [^{15}N]glycine (yield 58%) by the method of Schoenheimer & Ratner (1939).

Isolation of urinary hippuric acid and free urinary glycine (as 2:4-dinitrophenylglycine)

The urine samples were collected in plastic bottles and stored in a deep-freeze cabinet (−20°). Each specimen was concentrated to a volume of about 150 ml. by distillation under reduced pressure at 37° (this avoids hydrolysis of the urinary peptides) in an atmosphere of N_2. The pH of the concentrate was adjusted to about 1 by the addition of conc. HCl and extracted twice (in a continuous-extraction apparatus) with ethyl acetate. The first extraction lasted 45 min.; hippuric acid was isolated from this extract and recrystallized repeatedly from water until its m.p. and mixed m.p. agreed with those of an authentic sample of the acid. Sufficient material for duplicate mass-spectrometric analyses (at least 20 mg.) was obtained in almost all cases; where the amount of the final product was insufficient for this purpose, it was diluted with a known amount of unenriched hippuric acid and recrystallized. The second ethyl acetate extraction was continued for 4 hr. to remove the last traces of hippuric acid and some of the urinary pigments, and this extract was discarded. The pH of the aqueous phase was then adjusted to approx. 9·0 with 5 N-NaOH; the precipitate of phosphates which formed was removed by centrifuging, washed twice with water and discarded. The combined aqueous phase and washings were transferred to a three-necked flask and the NH_3 and amino compounds present were treated with excess of benzoyl chloride under the Schotten–Baumann conditions. A twofold excess of benzoyl chloride, calculated on the basis of the upper limit of the normal range of the published values for the urinary excretion of NH_3 and amino acids (Hawk, Oser & Summerson, 1949; King & Wootton, 1956), was employed to allow for possible diurnal variations in the amounts of these substances excreted in the urine. Special care was taken to provide effective stirring, cooling (0°) and pH control during the reaction. When the reaction was complete 5 N-NaOH was added until the pH was approx. 10·0, and the benzamide which had been formed by the reaction of the urinary NH_3 with the benzoyl chloride was removed by continuous extraction with ethyl acetate. The aqueous phase was acidified (pH 1·0) by the addition of conc. HCl and the mixed *N*-benzoylamino acids were removed by continuous extraction with ethyl acetate. This extract was distilled to dryness and the residue hydrolysed with 6 N-HCl for 24 hr. The hydrolyzate was distilled to dryness, the brown residue was dissolved in 150 ml. of water and benzoic acid removed by continuous ether extraction. The aqueous solution, which contained the free urinary amino acids, some urinary pigments and a little NH_3, was evaporated to dryness on a steam bath. The residue was dissolved in water and $NaHCO_3$ was added until the evolution of NH_3 ceased; the solution was again evaporated to dryness; the residue was redissolved in water and the addition of $NaHCO_3$ and evaporation was repeated to remove the last traces of NH_3. The final residue was transferred to a light-tight stoppered bottle with the minimum volume of water; a twofold excess (calculated on the basis of published data for the normal urinary free amino acid excretion) of 1-fluoro-2:4-dinitrobenzene dissolved in redistilled methanol (2 ml.) was added and the bottle shaken mechanically for 24 hr.

The subsequent chromatographic isolation of 2:4-dinitrophenyl (DNP)-glycine was based on the separation procedure described by Krol (1952) in connexion with the determination of glycine in small samples of proteins. The reagents used were prepared as described by this author, except where the contrary is specifically stated, and all manipulations were performed with the minimum exposure to light. The reaction mixture was transferred to a separating funnel and shaken with peroxide-free ether (50 ml.), and the ether extract was washed with portions of water (5 ml.) until the washings were colourless. The washings were added to the aqueous phase, the pH of which was brought to 1·0 by the addition of conc. HCl, and a second extraction with peroxide-free ether was carried out in a continuous-extraction apparatus and continued until no more yellow colour passed into the ethereal phase. The ethereal extract was dried by the addition of anhydrous Na_2SO_4, filtered off and the solvent removed by distillation. The mixed DNP-amino acids were dissolved in acetone and the solution was made up to 50 ml. A sample (0·1 ml.) of this solution was evaporated to dryness, the residue was dissolved in 0·5% $NaHCO_3$ soln. (100 ml.), the light absorption at 360 mμ was measured and the mixed DNP-amino acid content obtained from a calibration curve constructed with solutions of synthetic DNP-glycine. It is appreciated that this value may not be strictly accurate because the DNP-derivatives of the different amino acids and other yellow substances

present will have slightly different light-absorption maxima, but the value obtained provided a satisfactory guide to the number of chromatographic columns of the size described below which would be needed for a given separation.

Columns of buffered Celite 545 (Johns-Manville Co. Ltd., London, S.W. 1) (2 cm. × 20 cm., pH 6·05) were prepared essentially as described by Krol (1952). It was found that satisfactory separation of DNP-glycine was obtained provided that not more than 250 mg. of 'mixed DNP-derivatives' (above) were loaded on to any one column. The DNP-glycine formed an intense band (R_f 0·12–0·14), which was preceded by three fast-running bands; a fourth faint band was also sometimes apparent and just ahead of the DNP-glycine band. No difficulty was experienced in eluting the DNP-glycine band alone, provided that the initial development of the chromatogram was allowed to take place slowly (i.e. the eluate being collected at a rate of 0·5 ml./min.). The DNP-glycine-containing eluate was distilled to dryness *in vacuo* and the DNP-glycine crystallized from ethyl acetate–*cyclo*hexane (1:9). The yields of DNP-glycine in the present work varied between 19 and 65 mg./3 hr. urine sample. [Evered (1956) quotes values for the daily excretion of free urinary glycine which are equivalent to 37–64 mg. of DNP-glycine/3 hr.] The identity and purity of each sample isolated was confirmed by chromatography on paper with the *tert.*-amyl alcohol and propanol–light petroleum (100–120°) solvent systems described by Blackburn & Lowther (1951). DNP-glycine isolated in this way melted at 200–203°, unchanged by admixture with a synthetic sample of the derivative.

Preparation of nitrogen samples for mass-spectrometric analysis

Glycine and hippuric acid were digested by the normal Kjeldahl procedure and the NH_3 so formed was converted into N_2 by treatment with sodium hypobromite (Francis, Mulligan & Wormall, 1954). DNP-glycine is not completely digested by this procedure and the modification described by Elek & Sobotka (1926) and by Baker (1955) was employed. DNP-glycine (approx. 6 mg.) and pure glucose (approx. 150 mg.) were weighed in the digestion flask. Kjeldahl reagent (3 ml.) (Francis *et al.* 1954) was added and the mixture heated gently for 6 hr. At the end of this period the mixture was still black. The rate of heating was then considerably increased and continued for 17 hr. Titration of the NH_3 formed by this method indicated that all the N_2 in the sample had been converted into NH_3, but comparison of isotopic enrichments between [^{15}N]glycine, [^{15}N]hippuric acid and DNP-[^{15}N]glycine gave a value for DNP-glycine 3% higher than for the corresponding glycine or hippuric acid (Table 1).

Preparation of carbon dioxide samples for mass-spectrometric analysis

[1-^{13}C]Glycine was subjected to combustion to form CO_2 in a Pregl type of combustion tube, and the CO_2 was collected in an apparatus similar to that described by Bradley, Holloway & McFarlane (1954) for the preparation of $^{14}CO_2$ samples. Any oxides of nitrogen present were decomposed by the hot-wire treatment described by those authors. Carbon dioxide from the carboxyl group only was obtained from DNP-[1-^{13}C]glycine and from [1-^{13}C]hippuric acid by the acylative decarboxylation procedure described previously (Crawhall & Watts, 1959).

EXPERIMENTAL

The experiments were all performed on the same healthy male subject (weight 72 kg.; height 187 cm.). Each experiment was preceded by an overnight fast and complete emptying of the bladder. Frequent draughts of water were taken to promote diuresis.

Experiment 1. The subject took [^{15}N]glycine (339 mg., 36·2 atoms % excess of ^{15}N), dissolved in water, by mouth. Urine specimen 1 comprised all urine voided during the subsequent 6 hr. and urine specimen 2 comprised all urine voided between 6 and 24 hr. after the ingestion of [^{15}N]-glycine. No food was taken during the collection of specimen 1; the subject's usual diet was taken during the collection of specimen 2. The free glycine was isolated from each of these specimens and its ^{15}N content was measured.

Experiment 2. The subject took [^{15}N]glycine (100 mg., 36·2 atoms % excess of ^{15}N) by mouth. This was followed by the collection of six 6 hr. urine collections, and a further dose of [^{15}N]glycine (100 mg.) was taken at the beginning of each urine collection period. After 36 hr., 3-hr. urine collections were made, no further [^{15}N]glycine being taken during this period. The total protein intake was restricted to 40 g./day during the experimental period and the subject's meals were arranged so that the dietary protein intake was spread approximately uniformly over each 24-hr. period. The hippuric acid and free glycine were isolated from each urine specimen and their ^{15}N contents determined.

Experiment 3. A single dose of [1-^{13}C]glycine (344 mg., 49·3 atoms % excess of ^{13}C) and eight 3 hr. urine samples were collected during the subsequent 24 hr. Hippuric acid and the free glycine were isolated and their ^{13}C contents determined. The subject's diet was arranged as in Expt. 2.

RESULTS

Experiment 1. The results of this experiment (Table 2) demonstrated that sufficient enrichment could be obtained in the first glycine metabolic pool for mass-spectrometric analysis of the free urinary

Table 1. *Content of* ^{15}N *in nitrogen obtained from* [^{15}N]*hippuric acid and* 2:4-*dinitrophenyl-*[^{15}N]*glycine synthesized from the same batch of* [^{15}N]*glycine, the isotope content of which was determined simultaneously*

Compound	^{15}N (atoms % excess) Mean	Extreme range of values	No. of observations
[^{15}N]Glycine	0·688	0·685–0·693	3
2:4-Dinitrophenyl-[^{15}N]glycine	0·709	0·705–0·714	3
[^{15}N]Hippuric acid	0·680	0·674–0·688	3

Table 2. *Content of* ^{15}N *in the free urinary glycine after a single oral dose of* [^{15}N]*glycine (Expt.* 1)

The subject took [^{15}N]glycine (339 mg., 32·1 atoms % excess) orally.

Urine collection No.	Time after administration of [^{15}N]glycine (hr.)	^{15}N Content of the isolated 2:4-dinitrophenyl-glycine (atoms % excess)
1	0–6	0·308
2	6–24	0·036

glycine isolated as the 2:4-dinitrophenyl derivative from a 6-hr. urine collection. However, the isotope content of the isolated glycine decreased so rapidly that the isotope enrichment of the sample isolated from the urine collected between 6 and 24 hr. after the administration of the [^{15}N]glycine could only be measured with an error of ±10%, and it was apparent that either a single very large (and therefore unphysiological) dose, or multiple smaller doses, of the labelled amino acid would be needed for an investigation in which it was desired to keep the glycine in the first glycine metabolic pool measurably enriched with the isotope for a longer period.

The observed isotope enrichments represented the mean value for the ^{15}N content of the free urinary glycine during the time intervals concerned. As only two urine collections were made, the first glycine metabolic-pool size and the turnover rate of glycine in the pool could only be deduced if a simple exponential decay of the isotope content of the pool was assumed, and evidence bearing on the validity of such an assumption was obtained from the results of Expts. 2 and 3. We are indebted to Professor J. Rotblat for the following calculation.

If it is assumed that the glycine pool is a single-compartment system in equilibrium and containing some [^{15}N]glycine, then

$$C_t = C_0 e^{-\lambda t}, \qquad (1)$$

where C_t is the concentration of [^{15}N]glycine in the pool at time t and C_0 is the concentration of [^{15}N]-glycine immediately after the administration of the isotope. The length of time required for orally administered [^{15}N]glycine to come into equilibrium with the endogenous glycine is not known but can be assumed to be short compared with the time of collection of the first sample (6 hr.).

If urinary glycine is being excreted at a constant rate then

$$C_{\text{mean } 0-t_1} = \frac{C_0 \int_0^{t_1} e^{-\lambda t}\,dt}{t_1} = C_0 \frac{1-e^{-\lambda t_1}}{\lambda t_1} \qquad (2a)$$

and

$$C_{\text{mean } t_1-t_2} = \frac{C_0 \int_{t_1}^{t_2} e^{-\lambda t}\,dt}{t_2-t_1} = C_0 \frac{e^{-\lambda t_1}-e^{-\lambda t_2}}{\lambda(t_2-t_1)}, \qquad (2b)$$

where $C_{\text{mean } 0-t_1}$ is the mean value of concentration of [^{15}N]glycine in the pool and in the urine over the period $0-t_1$ and $C_{\text{mean } t_1-t_2}$ is a similar value found over the period t_1-t_2. Therefore

$$\frac{C_{\text{mean } 0-t_1}}{C_{\text{mean } t_1-t_2}} = \frac{1-e^{-\lambda t_1}}{e^{-\lambda t_1}-e^{-\lambda t_2}} \cdot \frac{t_2-t_1}{t_1}. \qquad (3)$$

In this experiment $t_1 = 6$ hr. and $t_2 = 24$ hr., so that $e^{-\lambda t_2}$ may be neglected by comparison with $e^{-\lambda t_1}$. Equation (3) can most easily be solved by employing this simplification. The values of $C_{\text{mean } 0-t_1}$ and $C_{\text{mean } t_1-t_2}$ have been found (Table 2). Therefore

$$\frac{0{\cdot}308}{0{\cdot}036} = \frac{t_2-t_1}{t_1}(e^{\lambda t_1}-1).$$

By substituting values of $t_1 = 6$ hr. and $t_2 = 24$ hr. it is found that

$$\lambda t_1 = 1{\cdot}36,$$

therefore

$$\lambda = 0{\cdot}23 \text{ hr.}^{-1},$$

which is equivalent to a half-life time of 3 hr. Substituting these values in equation (2a), $C_0 = 0{\cdot}566$. Therefore

$$\text{dilution of original [}^{15}\text{N]glycine fed} = \frac{10{\cdot}7}{0{\cdot}566} = 18{\cdot}9 \text{ times.}$$

This value of 10·7 atoms % excess of ^{15}N is the enrichment of fed glycine calculated as DNP-glycine, as that is the form in which it was isolated.

First glycine metabolic-pool size (0·339 × 18·9 g.) = 6·4 g. Turnover rate of glycine in pool ($\lambda \times$ pool size) = 1·73 g./hr. As the subject weighed 72 kg. this is a turnover rate of 0·32 m-mole/kg./hr

Experiment 2. This experiment was designed to determine whether a constant level of isotope enrichment in the first glycine metabolic pool could be achieved by the administration of multiple doses of isotopically labelled glycine and to compare the isotope contents of the urinary hippuric acid and the first glycine metabolic pool in this type of experiment. We also attempted to study the rate of disappearance of the isotope from these situations after the cessation of feeding with [^{15}N]glycine to test the validity of the assumption made in the calculation of pool size and the glycine-turnover rate from the data obtained in Expt. 1.

The average values for the ^{15}N contents of the urinary hippuric acid and the free urinary glycine were 0·546 (S.D. 0·124) atoms % excess and 0·261 (S.D. 0·040) atoms % excess respectively during the period of feeding with [^{15}N]glycine. It also appeared that the plateau value for the isotope enrichment of both substances was reached during the first 6 hr. of the experiment (Fig. 2). The isotope content of the DNP-glycine isolated from the urine specimens

collected after the cessation of feeding with [^{15}N]-glycine was too small for accurate mass-spectrometric analysis, so that no information could be obtained about the time course of the disappearance of the labelled amino acid from the free urinary glycine; this was partly due to the presence of two unenriched nitrogen atoms in the DNP-glycine molecule. The isotope content of the hippuric acid, which contains only the isotopically enriched amido group nitrogen atom, could be followed satisfactorily up to 69 hr. after the cessation of feeding with [^{15}N]glycine. These results, which have been plotted on a semilogarithmic scale in Fig. 3, indicate that the isotope enrichment is not decreasing in a simple exponential manner. Although the later points lie on a straight line, the gradient of which corresponds to a half-life time of 18·5 hr., the early points are too scanty to permit the resolution of the apparently composite portion of the curve.

Experiment 3. This experiment was designed to permit the calculation of the first glycine metabolic-pool size and turnover rate from the time course of the disappearance of an isotopic label from the free urinary glycine after a single oral dose of isotopically labelled glycine, without having to assume that we were dealing with a single-compartment system (cf. Expt. 1). We also compared the isotope contents of the free urinary glycine and the urinary hippurate. The isotope content of the free urinary glycine did not decrease as a single exponential function with time after a single dose of [1-^{13}C]glycine (Fig. 4); the first two hippuric acid values were considerably greater than the corresponding free urinary glycine values, but the subsequent results were closely similar (Table 3). In order to calculate the first glycine metabolic-pool size from these results it

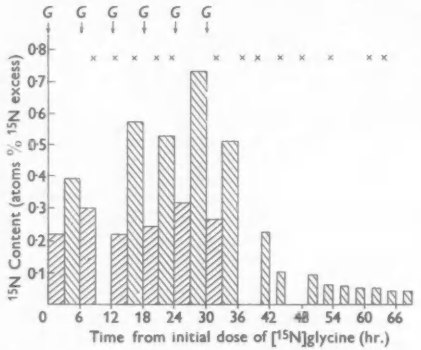

Fig. 2. Isotope content of the free urinary glycine (▨) and the urinary hippuric acid (▧) which resulted from repeated doses of [^{15}N]glycine (100 mg., 32·1 atoms % excess) given at the times indicated by *G*. Food was taken at the times indicated by ×. Further experimental details are described under 'Expt. 2' (Experimental section).

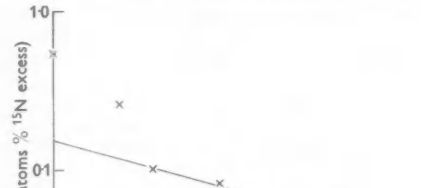

Fig. 3. Change with time of the isotope content of the urinary hippuric acid after the cessation of repetitive [^{15}N]glycine feeding (Expt. 2), plotted on semilogarithmic co-ordinates. Each point represents the average isotope content of the free urinary glycine for the 3 hr. period preceding the time opposite which it is plotted.

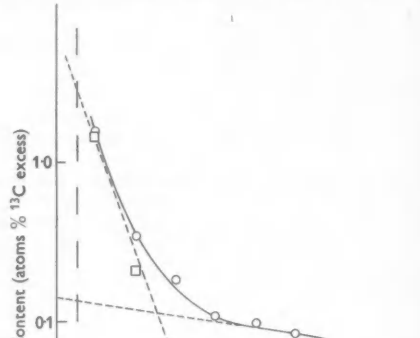

Fig. 4. Change with time of the isotope content of the free urinary glycine after a single oral dose of [1-^{13}C]glycine (344 mg., 49·3 atoms % excess of ^{13}C, Expt. 3) plotted on semilogarithmic co-ordinates. Each point represents the average isotope content of the free urinary glycine for the 3 hr. period preceding the time opposite which it is plotted. The resolution of the composite curve into two exponential components and the effective zero time (1·6 hr. after the ingestion of [1-^{13}C]glycine) are shown.

Table 3. *Content of* ^{13}C *in the free urinary glycine and the urinary hippuric acid after the administration of* [1-^{13}C]*glycine* (*Expt.* 3)

The subject took [1-^{13}C]glycine (344 mg., 49·3 atoms % excess) orally. Hippuric acid and 2:4-dinitrophenyl-glycine were isolated and degraded to yield their carboxyl group C atoms in the form of CO_2 as described in the Methods section.

Urine collection		Isotope content (atoms % excess) derived from carboxyl group only	
No.	Time after administration of [1-^{13}C]glycine (hr.)	2:4-Dinitrophenyl derivative	Hippuric acid
1	0–3	1·59	3·16
2	3–6	0·321	0·412
3	6–9	0·181	—
4	9–12	0·107	0·111
5	12–15	0·098	0·094
6	15–18	0·085	0·083
7	18–21	—	0·066
8	21–24	—	0·062

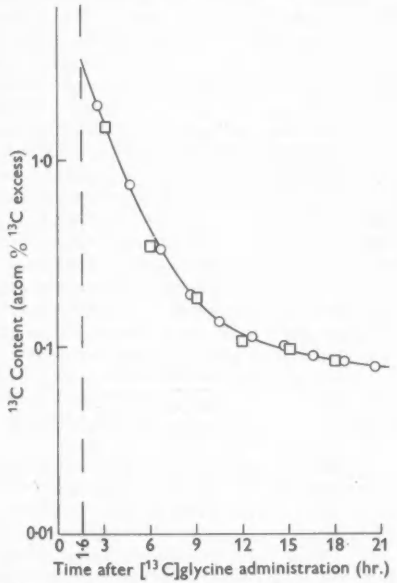

Fig. 5. Comparison of the observed ^{13}C content of the free urinary glycine (□) after a single oral dose of [1-^{13}C]-glycine (Expt. 3) with the corresponding value (○) calculated from the equation

$$C_t = 2{\cdot}95\, e^{-0{\cdot}552t} + 0{\cdot}16\, e^{-0{\cdot}0377t},$$

where C_t is the ^{13}C content of the free urinary glycine at time t. This time is 1·6 hr. greater than the experimental time, which was measured from the moment of [1-^{13}C]glycine ingestion.

would be desirable to know the isotope enrichment in the free urinary glycine at zero time. However, no such value is available because of the delayed mixing which occurs with administration by mouth, and because it is necessary to collect urine for 3 hr. to obtain sufficient DNP-glycine for the replicate mass-spectrometric analyses. More rapid mixing would have been obtained if the [^{13}C]glycine had been given intravenously, but this would have involved raising the plasma glycine to unphysiological levels. The values for the ^{13}C content of the free urinary glycine have been plotted in Fig. 4 at times corresponding to the end of each urine collection period, although they are in fact average values for each of the 3 hr. collection periods. It is possible to calculate a theoretical value for the initial isotope content (C_0) of the first glycine metabolic pool and hence to determine the effective zero time for the experiment in the following manner:

$$C_{mean} = \frac{C_0}{\lambda t}[1 - e^{-\lambda t}], \qquad (4)$$

where C_{mean} is the observed ^{13}C enrichment between 0 and 3 hr., from which the small contribution of the second exponential component has been deducted; C_0 is the theoretical initial ^{13}C enrichment which would have given rise to this mean value; λ is the exponential decay constant (0·552); t is the length of time covered by the collection period (i.e. 3 hr.).

Solution of this equation shows that the value of C_0 is 2·95 atoms % excess of ^{13}C. The administered glycine weighed 343 mg. and contained 49·8 atoms % excess of ^{13}C, so that it was diluted 16·9-fold between ingestion and its excretion as free urinary glycine, and the first glycine metabolic pool size is 5·8 g. By definition, the turnover rate of the pool [$\lambda \times$ pool size] is therefore 3·2 g./hr. (0·59 m-mole/kg. body wt./hr.) in this experiment. With the value of C_0 calculated from equation (4), it is found (Fig. 4) that the effective zero time of the experiment is 1·6 hr. after the experimental zero time (i.e. the time at which [^{13}C]glycine was administered). The C_0 value for the second exponential component of the curve based on the experimentally determined values (Fig. 4) is therefore 0·16 atom % ^{13}C

Table 4. *Comparison of experimentally determined values of the content of* ^{15}N *in free urinary glycine in Expt. 2 with corresponding calculated values*

Calculated values were obtained by application of equation (5), modified to allow for the different amounts of isotope administered in the two experiments.

Urine collection no.	^{15}N Content of free urinary glycine (atoms % excess)	
	Observed	Calculated
1	0·219	0·187
2	0·300	0·216
3	0·223	0·231
4	0·246	0·245
5	0·316	0·256
6	0·264	0·265

excess. By employing these two C_0 values and the observed slopes of the lines representing the two exponential rate processes, equation (5), which expresses the experimentally derived curve, can be calculated:

$$C_t = 2{\cdot}95\, e^{-0{\cdot}522t} + 0{\cdot}16\, e^{-0{\cdot}0377t}, \qquad (5)$$

where C_t is the ^{13}C content of the free urinary glycine at a time t, where $t = 0$ corresponds to 1·6 hr. on the experimental curve.

In Fig. 5, the curve derived from this equation has been plotted on semilogarithmic co-ordinates and the experimentally determined values have been superimposed.

The validity of equation (5) and hence of the deductions drawn from Expt. 3 is demonstrated by using this equation to calculate theoretical values for the isotope enrichment of the free urinary glycine during each of the 6 hr. urine-collection periods in Expt. 2. The agreement between the calculated and the experimentally determined values (Table 4) is considered to be satisfactory from this point of view.

The second exponential component of the curve shown in Fig. 4 has a half-life time of 16 hr. and corresponds to a second pool containing 107 g. of glycine with a turnover rate of 4 g./hr. The isotope disappeared from the urinary hippuric acid at a similar rate to this during the later parts of Expts. 2 and 3 (Fig. 3 and Table 3).

DISCUSSION

Size and turnover rate of the first glycine metabolic pool

The metabolic-pool concept is an abstract one and the term requires careful definition in the light of the context in which it is being used. In the present case, we wished to determine the amount of dilution which a dose of isotopically labelled glycine would undergo immediately after absorption and distribution. The amount of glycine with which the dose of labelled glycine would mix under these circumstances we term the 'first glycine metabolic pool'. This term was previously used by Arnstein & Neuberger (1951) to describe the rapidly turned-over glycine in the liver, kidney and intestine with which an injected dose of glycine would mix very quickly and which is in relatively slow equilibrium with the intracellular glycine of other tissues, with serine, glutathione, peptides and proteins. Henriques, Henriques & Neuberger (1955) obtained quantitative data for the turnover rate of glycine in a number of situations and concluded that, for the distribution of a labelled amino acid, a model consisting of a 'system of semi-isolated compartments communicating with a central compartment–the blood and extracellular fluid–at widely differing rates' approximates to the truth in mammals. Although there is no rigid proof that our first glycine metabolic pool corresponds exactly with the pool studied by Arnstein & Neuberger (1951) or with the 'central compartment' of Henriques *et al.* (1955), we feel that the correspondence is sufficiently close to make the introduction of yet another name unjustified. The most satisfactory source of a sample of the pool would probably be the free plasma glycine, but the published values for the glycine content of human plasma (or serum) vary between 11·6 and 39·0 μg./ml. (Arnstein, 1954), so that it was clearly impracticable to obtain sufficient amounts of glycine (at least 30 mg. of the DNP-derivative) for duplicate mass-spectrometric analysis from this source. The rationale underlying the use of the free urinary glycine as a sample of the first glycine metabolic pool has been explained in the introduction to this paper.

The results of Expt. 1 served to demonstrate that measurable amounts of the isotope would appear in the pool after a dose of labelled glycine which was small (339 mg.) relative to the approximate pool size (5·8 g.). It is clear from the results of Expt. 3 that, as was originally suspected, the assumption made in calculating the pool size and turnover rate from the data obtained in Expt. 1 was an over-simplification, the overall effect being to overestimate the pool size and to underestimate the turnover rate. The rate of disappearance of the isotope from the free urinary glycine in Expt. 3 corresponded well to an equation containing two exponential functions (Fig. 5), and the size and turnover rate of a 'second' as well as a 'first' glycine metabolic pool could in fact be calculated (see end of Results section). In view of the increasing multiplicity of factors (e.g. differential diffusion and reaction rates) which influence the distribution of the labelled amino acid, we do not think that speculation about the physiological significance or

the anatomical location of the second pool is justified. Henriques *et al.* (1955) found that after the intravenous injection of [^{14}C]glycine into a rabbit, the rate of loss of isotope from the plasma during the first 18 hr. after injection could be represented as the sum of three exponential processes. Although the method of graphical analysis which we have employed is a standard one in work of this type (see, for example, Robertson, 1957), it is open to the general criticism that it can be satisfactorily employed with many curves which do not in fact represent exponential functions. Also the number of components into which a given composite curve can be resolved may be influenced by the number of experimentally determined values which can be obtained for the parameter under discussion, and this may be limited in biological work. Thus it may be that the apparent difference between our results and that of Henriques *et al.* (1955) may result only from the smaller number of experimentally determined values upon which our curve is based.

In general the calculation of a pool size and hence of a turnover rate by an isotope-dilution technique presupposes that the labelled material mixes instantaneously with the pool at zero time. In our experiments, labelled glycine was given orally so that mixing could not be regarded as occurring instantaneously. This difficulty was overcome in Expt. 3 by calculating a value (C_0) for the isotope content of the glycine in the first glycine metabolic pool at a hypothetical zero time such that had the same dose of [^{13}C]glycine been administered at this hypothetical time and had it mixed instantly with the first glycine metabolic pool it would have given rise to the excretion pattern which we observed. Although errors of approximation are unavoidable in the mathematical treatment of these types of data, our values in man for the first glycine metabolic-pool size (1·07 m-moles/kg.) and turnover rate (0·59 m-mole/kg./hr.), which we found in Expt. 3, can be compared with some published values for small animals obtained in different types of experiment. Arnstein & Neuberger (1951) report a 'first glycine metabolic pool' size of 1·7 m-moles/kg. for the rat; in their experiments [^{14}C]glycine and sodium benzoate were injected into the peritoneal cavity. Turnover rates of 1·05 m-moles/kg./hr. (Arnstein & Neuberger, 1953) and 1·13 m-moles/kg./hr. Arnstein & Stanković (1956) have been reported for the rat, and a value of 1·66 m-moles/kg./hr. was found for the guinea pig (Arnstein & Stanković, 1956); these values were obtained by comparing the ^{14}C content of the dietary and visceral-protein glycine after prolonged feeding with [^{14}C]glycine.

When the isotope contents of the free urinary glycine and the urinary hippuric acid were compared during a period of repetitive [^{15}N]glycine feeding, values for the latter were consistently higher and more variable than for the former (Fig. 2). This suggests that the glycine which is used for hippurate synthesis is drawn preferentially from the portal blood stream as it enters the liver and before it has equilibrated with the first glycine metabolic pool. Simkin & White (1957) suggested that a large proportion of the glycine used for hippurate synthesis is provided by the free liver glycine; our findings lend further support to this idea.

Previous workers who have attempted to equate the isotope content of the glycine moiety of the urinary hippurate with that in the glycine metabolic pool have employed loading doses of benzoate to increase the urinary hippurate excretion. Stein, Paladini, Hirs & Moore (1954) isolated the urinary hippuric acid by ion-exchange chromatography and report that the normal adult male excretes 1·0–2·5 g. of this acid/24 hr. Armstrong, Chao, Parker & Wall (1955), who separated hippuric acid from urine by ethyl acetate extraction, likewise report excretion rates of 1–3 mg./kg. body wt./24 hr. for human subjects. These reports suggested that it might be possible to isolate sufficient amounts of hippurate for mass-spectrometric analysis without the use of loading doses of benzoate. This was possible in the majority of cases, although there were occasional failures. (Similar failures have not been encountered in the isolation of DNP-glycine.) We had hoped that when no loading dose of benzoate was given, and the possible disturbance of glycine metabolism which abnormal amounts of this ion itself might introduce (see, for example, Gray & Neuberger, 1950) was thereby avoided, the isotope content of the urinary hippurate would prove to be the same as that of the sample of the free glycine metabolic pool represented by the free urinary glycine. This was not so with urine samples obtained during or shortly after the administration of [^{13}C]-glycine (Fig. 2 and Table 3), although at time intervals more remote from the administration of the labelled amino acid the two sets of values agree (specimens 4–6 in Table 3). It is concluded that the isotope content of the glycine moiety of the urinary hippurate is not representative of that in the first glycine metabolic pool during or shortly after the administration of isotopically labelled glycine, and therefore that it could not be used as a means of sampling the pool under these conditions. It appears that at periods more than 9 hr. after the administration of isotopically labelled glycine (Table 3) the urinary hippuric acid could be used for this purpose, although further experimentation would be needed to determine the critical time interval exactly. This is comparable with the result of Arnstein & Neuberger's (1951) study, which

showed that the isotope content of the hippurate excreted by rats which were given benzoate after a dose of labelled glycine was only independent of the dose of benzoate when the interval between their administration was 6 hr. or longer.

SUMMARY

1. The amount of dilution which orally administered isotopically labelled glycine undergoes immediately after absorption and distribution has been investigated in man, and the 'pool' of glycine with which the labelled amino acid becomes diluted under these circumstances is termed the 'first glycine metabolic pool'.

2. The free urinary glycine (isolated as the 2:4-dinitrophenyl derivative) has been used as a sample of the first glycine metabolic pool in man. In experiments with [1-^{13}C]glycine and [^{15}N]glycine 3 hr. urine collections yield sufficient material for mass-spectrometric analysis.

3. The rate of change in the isotope content of the first glycine metabolic pool after a single oral dose of isotopically labelled glycine can be represented as the sum of two exponential functions. The first glycine metabolic-pool size and turnover rate of a 72 kg. adult were of the order of 5·8 g. (1·07 m-moles/kg. body wt.) and 3·2 g./hr. (0·59 m-mole/kg. body wt./hr.), respectively.

4. The isotope content of urinary hippurate and of the first glycine metabolic pool have been compared during a period when repeated doses of isotopically labelled glycine were being given and after a single oral dose of the labelled amino acid. It is concluded that the isotope content of the glycine moiety of the urinary hippuric acid even in the absence of loading doses of benzoate does not indicate the isotope content of the first glycine metabolic pool during or shortly after the administration of labelled amino acid although it may do so later.

We are pleased to acknowledge our indebtedness to Dr E. F. Scowen and to Professor A. Wormall, F.R.S. for their interest and encouragement during the prosecution of this work, to Professor J. Rotblat for his advice on mathematical matters and to Miss E. D. Bell and Miss L. E. Fraser for their skilled assistance.

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Liquid Scintillation-Counting for [^{14}C]Steroids

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The measurement of ^{14}C (β = 0·158 Mev) and of tritium (β = 0·018 Mev) is limited by the low energy of the emitted β particles. Standard techniques for the assay of these isotopes may be divided into the following three broad categories: (*a*) end-window and windowless flow gas counters operating in the proportional or Geiger region, which involve plating of the source at either infinite thinness or infinite thickness; (*b*) assay of the isotope in the gas phase after combustion of the biological sample; (*c*) liquid-scintillation techniques, of more recent introduction, in which the radioactive substance is incorporated into a liquid phosphor and the scintillations are 'observed' by means of a photomultiplier.

Method (*a*) suffers from the well-known difficulties in obtaining uniform and reproducible plating of the material to be assayed. Moreover, with plating techniques, it is considered necessary to convert the samples into a common form by combustion and incorporation into barium carbonate to obtain accurate and comparable specific radioactivities. Abrams & Clarke (1951) observed inaccuracies of 30% when attempting to count samples containing ^{14}C not subjected to combustion. However, Karnovsky *et al.* (1955) reported much smaller correction factors when counting samples, not subjected to combustion, by a windowless flow gas counter than have been reported for end-window Geiger counters. Where the latter method is employed the absorption of the low-energy β particles in the thinnest window available renders the method highly inefficient for ^{14}C assay and presents 'tremendous if not insurmountable difficulties for the assay of tritium' (Haigh, 1958). The estimation of a tritiated steroid in a methane flow proportional counter has, however, been described by Eidenoff & Knoll (1950). A windowless flow type of counter operating in the Geiger region for assay of low-energy β rays has been described by Damon (1951) and by Banks, Blow & Francis (1956), and applied to the estimation of tritium by Banks, Crawhall & Smyth (1956). Ayres, Pearlman, Tait & Tait (1958) have assayed tritiated progesterone and corticosteroids in a windowless flow counter.

Method (*b*), the assay of ^{14}C and tritium in the gas phase, overcomes the difficulties of plating the samples and the low sensitivity associated with end-window counters. Several methods have been described with ion chambers (e.g. Jesse, Hanum, Fostat & Hart, 1947); Janney & Moyer, 1947) and Geiger–Müller tubes (e.g. Brown & Miller, 1947; Bernstein & Ballentine, 1950; Glascock, 1952; Bradley, Holloway & McFarlane, 1954; Apelgot, Roumegous, Patureau & Moustacchi, 1955; Broda, 1955; Korshunov, Amenitskaya & Aĭvazov, 1955; Banks *et al.* 1956). These methods, although very sensitive and accurate, are time-consuming and unsuitable for routine use where large numbers of samples have to be assayed accurately, e.g. eluates from chromatographic columns. Method (*c*), the liquid scintillation-counting of ^{14}C and tritium, is gaining increasing popularity because of the high sensitivity and reproducibility due to a fixed 4π geometry and because of the elimination of window absorption, the necessity for plating and the elaborate technique required for gas counting. Many uses of the method have been described (e.g. Reynolds, Harrison & Salvini, 1950; Kallman & Furst, 1950; Bluh & Terentiuk, 1952; Farmer & Bernstein, 1952; Hayes & Gould, 1953; Rosenthal & Anger, 1954; Wagner & Guinn, 1955; Weinberger, Davidson & Ropp, 1956; White & Helf, 1956; Hodgson, Gordon & Ackerman, 1958). A balanced review has been given by Davidson & Feigelson (1957), who have discussed the advantages and disadvantages of liquid scintillation-counting in detail. The latest developments in this field are described by Bell & Hayes (1958).

The method has been applied to the estimation of ^{14}C-labelled steroid hormones (e.g. Werbin, Bergenstal, Gould & LeRoy, 1957; Chen, Schedl, Rosenfeld & Bartter, 1958; Kabara, Okita & LeRoy, 1958) and to some tritiated sterols by Hayes & Gould (1953), but there appears to be no systematic record of the variables affecting the method, especially as applied to this class of compound.

The advantages of liquid scintillation-counting for the detection of steroids are the high solubility of these compounds in the organic phosphor, the high overall efficiency and the convenience of the method. With the Ekco type N612 scintillation counter 65–69% efficiency has been obtained for the detection of ^{14}C (Table 1), based on [^{14}C]-naphth-2-ol and [^{14}C]testosterone sources obtained from The Radiochemical Centre, Amersham, Bucks. All counting was carried out at room temperature, under the optimum experimental conditions

described below, with a background of 1·5–2·0 counts/sec. There are a number of important experimental variables, however, which affect the absolute efficiency and the reproducibility of the method. It is thought that these variables may be of general application and that experiences in this respect may be of value to other workers in this and other fields.

APPARATUS AND MATERIALS

Apparatus. The Ekco type N612 Counter was connected to a stable power source (Harwell type 1007 or type 532B), timer (Harwell type 1003F), scaler (Harwell type 1009D) and decatron unit (Harwell type 1221c). The N612 counter uses a 13-stage photomultiplier, E.M.I. type 95145, with a simple linear amplifier offering alternative gains of 250, 500 and 1000 with an attenuation of 10 to 1 on these values if required. Details of its use and construction have been described by Haigh (1957).

Reagents. These must be purified to avoid possible ambiguities due to trace impurities. Ethyl acetate, light petroleum (b.p. 60–80°), acetone and methylene dichloride of A.R. grade were redistilled in Pyrex apparatus, the first and last 10% of the distillates being rejected. Chloroform and carbon tetrachloride of A.R. grade were fractionally distilled through a 150 cm. fractionating column. Benzene, A.R. grade, was redistilled twice, the first and last 10% of the distillates being rejected. Ethanol was dried and purified from volatile aldehydes and ketones by the method of Cook, Dell & Wareham (1955). Methanol, A.R. grade, was refluxed over CaO for 2 hr. and fractionally distilled through a 150 cm. fractionating column. Toluene of reagent grade, sulphur-free, was distilled three times in Pyrex apparatus, the first and last distillates being rejected in each case.

^{14}C-labelled compounds. [^{14}C]Testosterone and [^{14}C]-oestrone (The Radiochemical Centre, Amersham, Bucks.) were dissolved in benzene. [^{14}C]Naphth-2-ol (The Radiochemical Centre) was dissolved in toluene.

Organic phosphor. Scintipak (Nash and Thompson Ltd., Chessington, Surrey) was dissolved in the recommended quantity (4·2 g. in 1 l.) of purified toluene. The Scintipak consists of a mixture of 4 g. of *p*-terphenyl and 0·2 g. of *p*-bis-[2-(5-phenyloxazolyl)]benzene. Unless otherwise stated 'the scintillator' refers to the Scintipak solution described above.

EXPERIMENTAL

Effect of p-bis-[2-(5-phenyloxazolyl)]benzene on counting efficiency. No comparison of various primary and secondary solutes was made; these have been adequately described in the literature (e.g. Hayes, Ott, Kerr & Rogers, 1955; Hayes, Ott & Kerr, 1956). The efficiency of *p*-bis-[2-(5-phenyloxazolyl)]benzene in increasing the relative pulse height has been described by Hayes *et al.* (1956) with Dumont 6292 photomultipliers. That this compound is similarly effective with the E.M.I. 95145 photomultiplier is indicated from Fig. 1, which shows the increase in counting efficiency of the N612 counter when *p*-bis-[2-(5-phenyloxazolyl)]benzene is added to a primary solute of *p*-terphenyl in toluene. Although *p*-bis-[2-(5-phenyloxazolyl)]benzene and other secondary solutes may function as wavelength shifters, a report by Ziegler, Seliger & Jaffe (1955) indicated that the successful reversal of O_2 quenching may be one of the ways in which these compounds function. No allowance has been made for this quenching of the scintillator by dissolved O_2

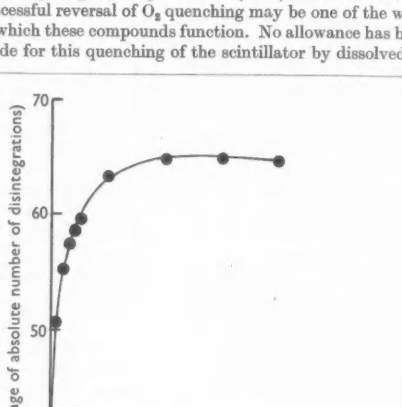

Fig. 1. Effect of *p*-bis-[2-(5-phenyloxazolyl)]benzene on the counting efficiency for [^{14}C]testosterone dissolved in 3 ml. of a solution of *p*-terphenyl in toluene (4 g./l.). Increasing amounts of *p*-bis-[2-(5-phenyloxazolyl)]benzene in toluene were added (2 g./l.).

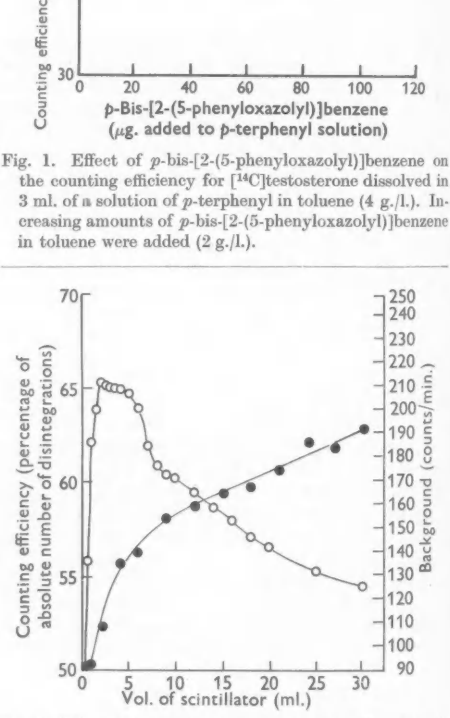

Fig. 2. Effect of increasing the volume of the phosphor, at constant concentration of scintillator, on the counting efficiency for a [^{14}C]testosterone source. ○, [^{14}C]Testosterone; ●, background count rate.

(Pringle, Black, Funt & Sobering, 1953; Arnold, 1954). Experiments were carried out to test the effects of bubbling N_2 (O_2-free) through the scintillator but no significant improvement in counting efficiency was obtained. Bubbling O_2 through the scintillator, however, reduced the counting efficiency by about 10%.

Variation of counting efficiency with volume of phosphor. Increasing volumes of the scintillator were added to a [14C]testosterone source and the count rate was observed at each volume (Fig. 2). The efficiency rose rapidly to an optimum which corresponded to a volume of 2–4 ml. of the scintillator. The efficiency thereafter decreased with increasing volume. The background count rose linearly with increasing volume of the scintillator (Fig. 2). For routine counting a volume of 3 ml. was selected.

Variation of counting efficiency with concentration of Scintipak in liquid phosphor. The efficiency of the count rose rapidly with increasing concentration of the Scintipak in the toluene (Fig. 3). For routine counting the concentration recommended by Nash and Thompson Ltd. (4·2 g./l.) was adopted.

Quenching of the scintillator. The two main problems in scintillation-counting are insolubility of the sample and quenching. The former is not troublesome in connexion with the steroid hormones but the latter was investigated at some length. The importance of solvents in liquid scintillators has been described by Hayes, Rogers & Sanders (1955) and the quenching of liquid scintillator solutions by a variety of organic compounds has been described by Kerr, Hayes & Ott (1957).

Effect of solvent impurities in the scintillator on the counting efficiency. In biochemical experiments with labelled compounds, the final material for assay may be presented in any of a number of various organic solvents following extraction from tissues or complex incubation systems. The effect of such solvents on the counting efficiency, when added to 3 ml. of the scintillator containing [14C]testosterone, can be seen from Fig. 4. The chlorinated hydrocarbons tested have a dramatic quenching effect, whereas solvents such as benzene, diethyl ether and light petroleum have effect only by dilution of the phosphor (Fig. 4). A wide range of solvents have been examined by Hayes, Rogers & Sanders (1955) for their effect on the relative pulse height from a liquid phosphor.

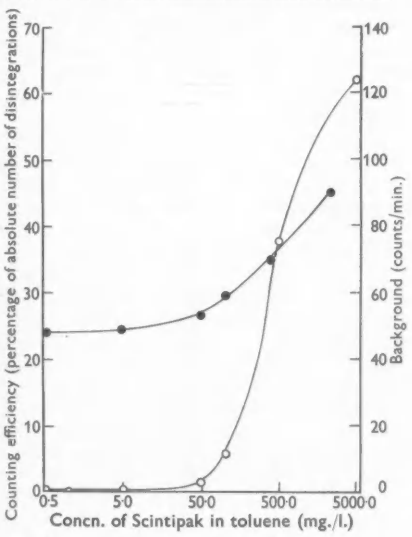

Fig. 3. Effect of the concentration of the scintillator at constant volume (3 ml.) on the counting efficiency. Scintipak of increasing concentration in toluene. ○, [14C]Testosterone sources (10^{-5} μc); ●, background.

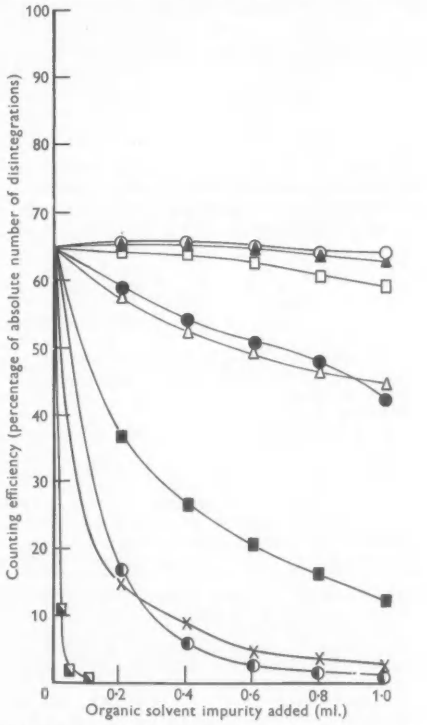

Fig. 4. Effect on the counting efficiency for [14C]testosterone of the addition to the scintillator (3 ml.) of some organic solvent impurities. ○, Benzene; ▲, diethyl ether; □, ethyl acetate; ●, ethanol; △, methanol; ■, methylene dichloride; ◐, chloroform; ×, acetone; ◺, carbon tetrachloride.

Table 1. *Efficiency of counter for* ^{14}C

[^{14}C]Naphth-2-ol and [^{14}C]testosterone sources were added either in solution to phosphor (3 ml.) or first evaporated to dryness in the counting pot before adding the phosphor (3 ml.).

^{14}C Source	Apparent counting efficiency (percentage of absolute number of disintegrations observed)	
	Evaporated in pot and redissolved	Added in solution to phosphor
Naphth-2-ol	58·0	65·0
	60·0	65·0
Testosterone	68·5	68·0
	68·5	68·5

When biological extracts are obtained in solution in a quenching solvent, this may be evaporated to dryness in the counting pot under infrared light. The sample may then be dissolved in the liquid phosphor and counted in the usual manner. A word of caution is necessary in this respect, however, since the ^{14}C source, especially when of high specific activity, may become partially adsorbed on the surface of the glass container. For such adsorbed material, 2π counting geometry may operate only, with a consequent loss of counting efficiency (Table 1).

Self-quenching from steroid hormones. To determine the suitability of the liquid scintillation method for the general assay of labelled steroids it was necessary to determine if self-quenching occurred with any of a large variety of steroids. The compounds listed in Table 2 were dissolved (1 mg./ml.) in benzene or benzene–diethyl ether (1:1, v/v) and 0·5–1·5 ml. of the solution of each steroid was added to the scintillator containing a standard [^{14}C]testosterone source. The decrease, if any, in absolute counting efficiency is shown in Table 2.

DISCUSSION

The liquid scintillation method has been found very convenient for the assay of labelled steroids because of their ready solubility in the organic phosphor. The method is very reproducible when attention is paid to the experimental factors given above and to some additional factors given below. The results given in Table 2 show that the method is of general

Table 2. *Effect of quenching by steroids on the counting efficiency for* ^{14}C

The steroid was dissolved* (1 mg./ml.) in benzene or benzene–diethyl ether (1:1, v/v) and 0·5, 1·0 and 1·5 ml. of the solution was added to the phosphor (3 ml.) containing [^{14}C]testosterone. The addition of 1·5 ml. of pure benzene or ether to the phosphor reduces the absolute counting efficiency by approx. 1·2 and 1·8%, respectively. The counting efficiency without added steroid solution was 65%.

Steroid added	Counting efficiency after addition of steroid (percentage of absolute number of disintegrations)		
Amount added (mg.) ...	0·5	1·0	1·5
Prednisone (17α:21-dihydroxypregna-1:4-diene-3:11:20-trione)*	64·5	64·1	63·1
9α-Fluoro-11β-hydroxy-17-methyltestosterone*	64·0	63·7	62·9
3α:17β-Dihydroxy-5α-androstane*	64·3	63·2	62·6
3α:17β-Diacetoxyandrost-5-ene	64·2	63·6	62·5
9α-Fluorocortisone acetate (9α-fluoro-17α-hydroxy-21-acetoxypregn-4-ene-3:11:20-trione)*	63·9	63·2	62·5
Hydrocortisone (11β:17α:21-trihydroxypregn-4-ene-3:20-dione)	64·1	63·1	62·3
3β:17α:21-Trihydroxypregn-5-en-20-one*	63·8	63·1	62·2
Oestradiol (3:17β-dihydroxyoestra-1:3:5(10)-triene	64·1	63·0	62·0
Androsterone (3α-hydroxy-5α-androstan-17-one)	64·0	62·7	61·9
Cholesterol (3β-hydroxycholest-5-ene)	64·0	62·9	61·9
Cortisone (17α:21-dihydroxypregn-4-ene-3:11:20-trione)*	64·0	63·0	61·8
3α:17α-Dihydroxy-5β-pregnan-20-one)	64·1	62·8	61·8
9α-Fluoro-11β-hydroxytestosterone (9α-fluoro-11β:17β-dihydroxyandrost-4-en-3-one)*	63·9	63·2	61·8
Testosterone (17β-hydroxyandrost-4-en-3-one)	64·2	62·9	61·8
5α-Androstane-3:17-dione	64·8	63·2	61·7
Oestriol (3:16α:17β-trihydroxyoestra-1:3:5(10)-triene)*	64·5	63·7	61·6
Compound S (17α:21-dihydroxypregn-4-ene-3:20-dione)	63·9	63·0	61·4
Progesterone (pregn-4-ene-3:20-dione)	64·3	62·6	61·3
Cholest-4-en-3-one	63·8	62·2	61·2
Pregnenalone (3β-hydroxypregn-5-en-20-one)	64·2	62·8	61·2
Androst-4-ene-3:17-dione	63·9	62·4	60·7
Androsta-1:4-diene-3:17-dione	63·9	62·3	60·4
Oestrone (3-hydroxyoestra-1:3:5(10)-trien-17-one)	62·7	62·0	60·2
5α-Androst-1-ene-3:17-dione	63·8	61·6	59·9
Equilenin (3-hydroxyoestra-1:3:5(10):6:8-pentaen-17-one)	63·0	61·0	58·7

* Steroids were added in slight suspension owing to partial insolubility in the solvents used.

application for the assay of ^{14}C-labelled steroids, since strong quenching of the scintillator was not caused by any of the 25 compounds tested. Definite quenching was, however, shown by certain steroids, notably oestrone and equilenin. There appears to be no systematic relationship between structure and quenching potential with most of the compounds tested. The compounds listed in Table 2 were tested only to a concentration of 1·5 mg. in the scintillator. Where assays are to be made of compounds of low specific activity, however, where the carrier is in excess of 1·5 mg., then further tests would be necessary at the appropriate concentration. Correction for quenching should be made for any compound where its concentration in the scintillator may have a significant effect. Caution should be exercised also when using the method for the assay of crude biological extracts where interfering or quenching compounds or colour (Kinnory *et al.* 1958) may accompany the radioactive product under investigation.

It is a simple matter to test such extracts by addition of a source of accurately known value and to make the appropriate correction for suppression of the expected count.

The Ekco N612 counter uses a small (46 mm. diameter) counting pot. Optical contact of the base of this pot with the top of the photomultiplier is made with silicone oil (Hopkin and Williams Ltd.; MS 200/2 centistokes). The silicone fluid (MS 200/20 centistokes) recommended earlier by the manufacturer (E. K. Cole Ltd.) was less satisfactory since bubbles tended to be trapped beneath the counting chamber in this oil of higher viscosity. Despite the fact that these chambers are manufactured with slightly convex bases it is still possible to trap an occasional bubble beneath the vessel, especially when the silicone fluid is low. It is necessary to pay particular attention to the elimination of all such bubbles, since they have a marked effect on the counting efficiency and thereby upon the reproducibility of the method. In this connexion it should be pointed out that this silicone oil does, in time, become contaminated with atmospheric dust and dirt and extraneous matter conveyed in with the counting vessel. In initial experiments the vessels were carefully wiped with paper tissue to remove dirt and in later experiments with a lens tissue. In both cases reproducibility of the method was unsatisfactory and this was traced to the accumulation, during several weeks' use, of particles of the paper or lens tissue in the silicone fluid. In addition a minute amount of swarf or particles of paint worked into the oil initially and may have added to this trouble. This sort of contamination of the oil gives rise to two effects: (*a*) an intermittent lack of reproducibility due to the disturbance of the contaminating particles every time the shutter mechanism is operated in the silicone oil and (*b*) a long-term increase in interfering material which is reflected in a gradual reduction of the observed count from a standard source.

To overcome these difficulties a chamois leather is now used for wiping the counting vessels before they are introduced into the counter and, most important, a ^{14}C standard test source is maintained. This is of the order of 2×10^4 counts/min., and assay at regular intervals reveals accumulation of contaminating dust necessitating a change of the silicone fluid.

A great deal has been said about possible interference from fluorescence of the glass counting chambers. Under the conditions employed for determination of ^{14}C, however, we have failed to induce deliberately any significant fluorescence effects into these containers either by sunlight or fluorescent lighting.

If attention is paid to the details mentioned above, liquid scintillation-counting is regarded as the most convenient method for the determination of ^{14}C-labelled steroids, despite the rather high background as compared with other methods. Under the optimum experimental conditions described in this paper, greater efficiency (65–69 %) has been obtained than can be achieved by end-window or windowless flow gas counters, and much greater convenience than with apparatus for the determination of this isotope in the gas phase. The reproducibility and the efficiency with the scintillation technique at room temperature are sufficiently high for all practical purposes and the added expense and inconvenience of refrigeration would not seem justified for assay of ^{14}C.

SUMMARY

1. The assay of ^{14}C-labelled steroids with the Ekco type N612 liquid scintillation counter is described.

2. The effects of scintillator volumes and concentration on the efficiency of the method are given.

3. Self-quenching by 25 different steroids has been examined. None of the steroids, at the levels tested, exerted strong quenching effects, although slight quenching was apparent with some, in particular with equilenin and oestrone.

4. The effect of impurities in the scintillator on the efficiency of the method has been investigated.

5. Some additional variables which affect the reproducibility are discussed.

The author is indebted to Mrs Diana Gardner for technical assistance during the course of this work, to Mr R. Hunt for services to the electronic equipment and to Dr R. I. Dorfman, Worcester Foundation for Experimental Biology, Shrewsbury, Mass., U.S.A. for gifts of steroids.

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Studies on Carbohydrate-Metabolizing Enzymes

2. TRANS-α-GLUCOSYLATION BY EXTRACTS OF *TETRAHYMENA PYRIFORMIS**

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In continuation of studies on transglucosylation reactions (Duncan & Manners, 1958; Anderson & Manners, 1959; Duncan, Manners & Thompson, 1959) we now describe an investigation of the metabolism of maltose by cell-free extracts of the ciliate *Tetrahymena pyriformis*. The presence of a maltase in such extracts has been noted by Ryley (1952).

Since maltase activity may be regarded as a trans-α-glucosylation reaction in which water provides the acceptor substrate:

$$\text{Maltose} + \text{enzyme-H} \rightarrow \text{glucose} + \text{glucosyl-enzyme}$$
$$\text{Glucosyl-enzyme} + \text{H·OR} \rightarrow \text{enzyme-H} + \text{glucosyl·OR}$$

where H·OR is the glucosyl acceptor (Gottschalk, 1958), it follows that, if oligosaccharide synthesis takes place in concentrated solutions of maltose,

* The paper by Anderson & Manners (1959) is regarded as Part 1.

the nature of the products provides information on the specificity of the maltase for the acceptor H·OR. This has been obtained in the present study by chromatographic separation and characterization of the oligosaccharides synthesized from 18% maltose solution. In addition, the ability of other sugars to function as acceptors has been examined with various maltose–monosaccharide mixtures.

MATERIALS AND METHODS

Analytical methods. The methods used have been described by Duncan & Manners (1958) and Anderson & Manners (1959). An ethyl acetate–pyridine–water (10:4:3, by vol.) mixture was used as solvent for paper chromatography.

Organism. A pure culture of *Tetrahymena pyriformis* (GL) was kindly supplied by Dr J. F. Ryley and was maintained at room temperature in a sterile medium, adjusted to pH 7·3, containing 1% (w/v) of Oxoid brand bacteriological peptone and 0·4% (w/v) of sodium chloride. Sub-inoculations were made at intervals of 2 weeks (Ryley, 1952).

For large-scale culture, the organism was grown in the dark at 30° in 2 l. conical flasks containing 400 ml. of medium. Maximum culture density was reached after 6 days. The contents of twenty-two such flasks were combined and the cells collected by using a Sharples super-centrifuge. The cells were then dispersed in 150 ml. 0·1 M-sodium citrate buffer (pH 6·0) and freeze-dried to give 7·0 g. of yellow–brown powder. Microscopic examination of an aqueous suspension of this material showed that the cells had disintegrated. Part of the freeze-dried powder (0·35 g.) was suspended in 10 ml. of water and kept, with frequent stirring, at room temperature for 1 hr. Centrifuging at 3500 *g* yielded a yellow, opalescent solution which was free from cells and cell particles.

A further portion of freeze-dried cells (approx. 7 g.) was extracted with 200 ml. of water and centrifuged, and the extract was then freeze-dried (yield, 4·8 g.). This material was stored at 2° over calcium chloride; the maltase activity was not appreciably diminished after 1 year. However, the following experiments were carried out within 4 weeks of preparation of the extract.

The possibility of reducing sugars' being present in this extract, or arising by autolysis of soluble polysaccharide, was examined by paper-chromatographic analysis of a 4% (w/v) aqueous solution during incubation at 35° for 5 days. A faint trace of glucose was detected initially; this did not increase.

EXPERIMENTAL AND RESULTS

Carbohydrase activity of extract

Digests containing carbohydrate (2%, w/v) and extract (2%, w/v) were incubated at 35° for 24 hr. The following carbohydrates were hydrolysed: maltose, isomaltose, panose, maltotriose, starch, cellobiose, sucrose, methyl α-D-glucoside, α-D-glucosyl phosphate. Lactose was not attacked. In view of the considerable α-glucosidase activity, the action was investigated with a concentrated solution of maltose (*a*) alone, (*b*) in the presence of possible α-glucosyl acceptors.

Action on concentrated solution of maltose

A digest containing maltose (20%, w/v) and extract (1%, w/v) was incubated at 35° in the presence of toluene. A number of oligosaccharides were produced, as shown in Table 1.

Preparation and isolation of oligosaccharides

Thrice-recrystallized and chromatographically-pure maltose) 30·0 g.), 150 ml. of 0·1 M-sodium citrate buffer (pH 6·0) and 20 ml. of enzyme extract were incubated, with toluene, at 35° for 10 days. The enzyme extract was prepared from 3·8 g. of freeze-dried cells, which were dispersed in 60 ml. of water at room temperature for 2 hr. and then centrifuged (3500 *g* at 2°). Paper-chromatographic analysis at intervals gave the same results as those shown in Table 1. The digest was then heated for 20 min. at 98°, and filtered.

The oligosaccharides were separated by chromatography on a charcoal–Celite column (160 × 7·5 cm.) and elution with increasing concentrations of aqueous ethanol. Fractions (1–2 l.) were collected, concentrated to approx. 5 ml., freed from traces of charcoal and Celite and examined by paper chromatography. Fractions containing like components were combined, and the sugars isolated in the usual manner by crystallization from ethanol or by freeze-drying. The approximate yields are given in Table 2. All the fractions were homogeneous by paper chromatography and electrophoresis, and on acid hydrolysis gave only glucose.

Characterization of oligosaccharides

Fraction 1. The data in Table 2 and the specific rotation in water (+52°) characterize this sugar as D-glucose.

Fraction 2. Partial and total acid hydrolysis gave glucose; emulsin had no action. This disaccharide is therefore isomaltose.

Table 1. *Oligosaccharides synthesized from maltose by cell-free extracts of* Tetrahymena pyriformis

+++, ++, + and *t* indicate intense, strong, moderate and weak spots, respectively. Glucose, maltose, isomaltose, maltotriose and panose had R_G values of 1·00, 0·62, 0·47, 0·39, 0·28, respectively.

Time of incubation (days)	R_G values					
	1·00	0·62	0·47	0·39	0·28	Others
2	++	+++		+	+	*t*
4	++	+++	*t*	+	+	*t*
6	++	+++	+	+	+	+
8	+++	+++	+	++	++	+
10 and 15	+++	+++	++	++	++	+

Fraction 3. This was identified as maltose by specific rotation (+132°), partial acid hydrolysis and the data in Table 2.

Fraction 4. This trisaccharide had the same R_G as authentic panose and $[\alpha]_D + 148°$ in water (*c*. 2·3); cf. $[\alpha]_D + 148°$ reported for panose by Duncan & Manners (1958). A partial acid hydrolysate contained glucose, maltose and isomaltose; the corresponding aldonic acid, prepared by hypoiodite oxidation, gave glucose and isomaltose. On periodate oxidation, 0·94 mol.prop. of formaldehyde was released. The theoretical value is 1·00 mol.prop.

Fraction 5. This trisaccharide was characterized as maltotriose by R_G value, $[\alpha]_D + 161°$ in water (*c*, 2·7) [cf. $[\alpha]_D + 160$ and $+164°$ quoted by Whelan, Bailey & Roberts (1953) and Duncan & Manners (1958), respectively] and slow hydrolysis to glucose and maltose by salivary α-amylase. Partial acid hydrolysates of the sugar and the corresponding aldonic acid both contained glucose and maltose.

Fraction 6. This sugar was a tetrasaccharide with R_G 0·18 and $[\alpha]_D + 172°$ in water (*c*, 0·4); cf. values of R_G 0·17 and $[\alpha]_D + 177°$, reported by Duncan & Manners (1958). Partial hydrolysis with acid gave glucose, maltose, isomaltose, panose and maltotriose. The corresponding aldonic acid gave the same products on partial hydrolysis, except that maltotriose was absent. This finding eliminates 6^1- and 6^2-α-glucosylmaltotriose [see Crook & Stone (1957) for definition of nomenclature] as possible structures. The characterization as the 6^3 isomer is confirmed by the periodate oxidation (Table 2).

Fraction 7 (with Miss Zeenat H. Gunja). This tetrasaccharide had $[\alpha]_D + 180°$ in water (*c*, 0·9); cf. $[\alpha]_D + 177°$, quoted by Whelan *et al.* (1953) for maltotetraose. Partial hydrolysis with acid gave glucose, maltose and maltotriose. α- and β-Amylolysis gave a mixture of glucose and maltose, and maltose, respectively.

Trans-α-glucosylation to other sugars

Digests containing maltose (5 %) and a pentose, D-galactose, or D-fructose (5 %) and enzyme extract (1 %) were prepared and incubated at 35°. After 3–5 days' incubation the presence of new components in addition to isomaltose, maltotriose and panose was detected by paper chromatography. The new sugars had R_G values of 0·64 (D-xylose acceptor), 0·69 (D-lyxose acceptor), 0·64 (D-ribose acceptor), 0·72 (L-arabinose acceptor), 0·32 (D-galactose acceptor). The new sugars formed in the digests containing pentoses gave pink stains with aniline oxalate and are presumably α-glucosylpentose disaccharides. Fructose did not function as an acceptor substrate.

Other transglucosylation reactions

Various carbohydrates (20 % solution) and extract (2 %) were incubated for 5 days at 35°. The following results were obtained by paper chromatography: (*a*) isomaltose: products had R_G 1·0, 0·47, 0·19 (? isomaltotriose); a trace of higher oligosaccharides was also present; (*b*) methyl α-D-glucoside: the main product had R_G 1·0; a trace of higher oligosaccharides was produced; (*c*) D-glucose, (*d*) D-xylose, (*e*) α-D-glucosyl phosphate: no synthesis of oligosaccharides.

DISCUSSION

The ability of maltase preparations from a variety of biological sources to catalyse the synthesis of oligosaccharides from maltose has been reported by many workers (see Duncan & Manners, 1958).

The present results (Tables 1 and 2) show that cell-free extracts of the ciliate *Tetrahymena pyriformis* have a similar activity. On incubation with 18 % maltose solution, the main products are panose and maltotriose; smaller quantities of isomaltose, 6^3-α-glucosylmaltotriose and maltotetraose are also formed. Since the extracts are unable to polymerize glucose [in contrast with certain fungal preparations (Peat, Whelan & Hinson, 1955)], it follows that the oligosaccharides are formed by transglucosylation in which the OH groups at both C-4 and C-6 of the non-reducing residue of maltose and maltotriose can function as acceptors for α-glucosyl

Table 2. *Properties of the oligosaccharides*

The degree of polymerization was determined from reducing-power measurements before and after acid hydrolysis. The periodate oxidation value is expressed as moles of formaldehyde per mole of oligosaccharide liberated on oxidation at pH 8. With fractions 5 and 6 a small amount of sugars with R_G 0·31 and 0·15 was also eluted with 8 % (v/v) ethanol.

Fraction no.	Eluent	Yield (g.)	R_G	Degree of polymerization	Periodate oxidation value	Identity
1	Water	—	1·00	1·00	1·01	Glucose
2	Water	0·41	0·47	1·90	0·08	Isomaltose
3	Ethanol (2–6 %, v/v)	5·96	0·62	2·10	1·70	Maltose
4	Ethanol (6 %, v/v)	2·94	0·28	3·20	0·94	Panose
5	Ethanol (6–8 %, v/v)	3·29	0·41	2·94	2·80	Maltotriose
6	Ethanol (8 %, v/v)	0·58	0·18	3·70	1·98	6^3-α-Glucosylmaltotriose
7	Ethanol (10–12 %, v/v)	0·35	0·26	4·08	—	Maltotetraose

radicals. Panose does not appear to be a favourable acceptor, since 6^2-α-isomaltosylmaltose and related sugars are not produced. The ability to transfer to C-4 differentiates the protozoal enzyme system from various fungal maltases which can only synthesize (1 → 6)-linked oligosaccharides (e.g. Pazur & French, 1952).

The acceptor specificity of the protozoal maltase system is generally similar to that observed with extracts of *C. rupestris* (Duncan & Manners, 1958) except that the latter is unable to use monosaccharides as acceptor substrates. Since a number of pentoses and also D-galactose can accept α-glucosyl radicals with the *T. pyriformis* system, it is probable that most of the isomaltose is formed by the interaction of a glucosyl–enzyme complex with the primary alcohol group of a glucose molecule rather than by the hydrolysis of the (1 → 4)-linkage of panose. This view is supported by the fact that isomaltose synthesis and the formation of higher oligosaccharides (R_g < 0·28) occur simultaneously, showing that the equilibrium between hydrolysis (i.e. water as glycosyl acceptor) and trans-α-glucosylation to maltotriose favours the latter.

Isomaltose itself can function as a glucosyl donor, but the product appears to be isomaltotriose rather than panose or a maltosaccharide. One possible explanation for this is that the maltase and isomaltase activities of the *T. pyriformis* extracts are due to different enzymes. The non-identity of these activities in extracts of yeast and intestinal mucosa (for example, Larner & Gillespie, 1956) has already been established.

SUMMARY

1. Cell-free extracts of the ciliate *Tetrahymena pyriformis* show hydrolytic activity towards maltose, isomaltose, cellobiose, sucrose, methyl α-D-glucoside, α-D-glucosyl phosphate and starch.

2. In concentrated solution, oligosaccharides are synthesized from maltose and from isomaltose, but not from glucose.

3. Oligosaccharide synthesis from maltose results in the formation of isomaltose, maltotriose, panose, 6^2-α-glucosylmaltotriose and maltotetraose.

4. The acceptor specificity for α-glucosyl radicals by the protozoal enzyme system and other maltases is discussed.

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Enzyme Systems in Marine Algae

3. TRANS-β-GLUCOSYLATION BY EXTRACTS OF *CLADOPHORA RUPESTRIS* AND *ULVA LACTUCA**

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During a study of the hydrolysis of cellodextrin by an extract of *Cladophora rupestris*, Duncan, Manners & Ross (1956) noted that the products of enzyme action, as examined by paper chromatography, included laminaribiose. Since the original substrate did not contain β-1:3-glucosidic linkages, it seemed possible that this sugar had arisen from cellobiose by trans-β-glucosylation involving a double-displacement mechanism:

$$\text{Cellobiose} + \text{enzyme-H} \rightarrow \text{glucose} + \text{glucosyl-enzyme}$$

$$\text{Glucosyl-enzyme} + \text{H·OR} \rightarrow \text{enzyme-H} + \text{glucosyl·OR}$$

where RO·H is an acceptor substrate, in this case D-glucose acting at its C-3 hydroxyl group.

* Part 2. Duncan & Manners (1958).

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This possibility has now been investigated by incubating the algal extract with various concentrations of cellobiose, and with mixtures of cellobiose and other sugars. In particular, the ability of D-xylose to function as a β-glucosyl acceptor has been studied. While this work was in progress, Barker, Bourne, Hewitt & Stacey (1957) reported that cell-free extracts of *Aspergillus niger* (strain 152) could synthesize 3-*O*-β-D-glucopyranosyl-D-xylose from a cellobiose–xylose mixture.

The metabolism of cellobiose by an extract of *Ulva lactuca* has also been examined.

METHODS

Chromatography. (*a*) *Qualitative.* Descending paper chromatograms were prepared at room temperature with Whatman no. 1 or 54 paper and butanol–pyridine–water–benzene (5:3:3:1, by vol.) (*A*), ethyl acetate–acetic acid–formic acid–water (18:3:1:4, by vol.) (*B*) or ethyl acetate–pyridine–water (10:4:3, by vol.) (*C*) as solvent. Aniline phthalate or oxalate, *p*-anisidine hydrochloride, naphtharesorcinol–hydrochloric acid and silver nitrate–sodium hydroxide were used as spray reagents. The rate of movement of sugars is expressed relative to D-glucose (R_G) or to D-xylose (R_X).

(*b*) *Preparative.* Charcoal–Celite columns were prepared as previously described (Duncan & Manners, 1958).

Oxidation by periodate. The reduction of periodate was determined spectrophotometrically (Aspinall & Ferrier, 1957). The remaining methods were those described by Duncan & Manners (1958) and Anderson & Manners (1959).

Enzyme preparations. For the preliminary experiments, unfractionated extracts of *C. rupestris* and *U. lactuca* were used (Duncan *et al.* 1956). In the large-scale digest, an extract of *C. rupestris* was fractionated with ammonium sulphate and the protein precipitated between 0·18 and 0·36 saturation was collected, dissolved in water, dialysed against running tap water for 24 hr. at room temperature and freeze-dried. This fraction had the highest β-glucosidase activity. Toluene was used to maintain aseptic conditions in the enzymic reactions.

EXPERIMENTAL AND RESULTS

C. rupestris *extract*

Action on cellobiose. Digests containing cellobiose (1, 5, 10, 20 %, w/v) and *C. rupestris* enzyme preparation (1 %, w/v) were incubated at 35°. In all digests, glucose was detected after 3·5 hr. The other results were as follows: (*a*) 1 % cellobiose, hydrolysis complete within 96 hr.; (*b*) 5 % cellobiose, oligosaccharides detected after 96 hr.; (*c*) 10 and 20 % cellobiose, oligosaccharides produced within 24 hr. These were identified, by paper chromatography in three solvents, as laminaribiose, gentiobiose and cellotriose. The cellotriose was formed first, and in greatest amount; we are indebted to Dr W. J. Whelan for an authentic sample for comparison.

In a further experiment, cellobiose (6 %, w/v) and extract were incubated for 14 days. In addition to the above oligosaccharides, three sugars with R_g values of 0·12, 0·08 and 0·06 were also produced (solvent *A*).

Since these results were generally similar to those reported with β-glucosidase preparations from various moulds and plants (Crook & Stone, 1957) and, in particular, from barley in which the identity of the laminaribiose and gentiobiose was confirmed by chemical methods (Anderson & Manners, 1959), it was decided to examine the acceptor specificity for β-glucosyl residues in greater detail.

Action on cellobiose–sugar mixtures. Digests were prepared containing cellobiose (15 %, w/v) with D-xylose, L-xylose, L-sorbose or N-acetyl-D-glucosamine (15 %, w/v) and enzyme extract (1 %, w/v) and were incubated at pH 5·6 and 35° for 10–15 days. The last two digests contained the same series of sugars as with cellobiose alone. The pentose-containing digests contained additional sugars which stained pink with aniline oxalate. In solvent *B*, those from the D-xylose digest had R_G values 0·70 and 0·40; in the L-xylose digest, R_g 0·72 and 0·42. The sugars with R_G 0·70 and 0·72 were eluted from paper chromatograms with cold water, hydrolysed with sulphuric acid, neutralized and re-chromatographed. Each hydrolysate contained glucose and pentose in apparently equivalent quantities, and no other sugar. The sugars with R_g 0·40 and 0·42, which were present only in small amounts, were similarly examined; both yielded glucose and pentose. It is possible that these sugars are diglucosylxylose trisaccharides.

The presence of more than one new component in the pentose digests was confirmed by chromatography on small charcoal–Celite columns. Elution with water gave a mixture of monosaccharides, with 5 % aqueous ethanol yielded cellobiose, and with 10 % ethanol the following: with D-xylose as acceptor, R_g 0·74, 0·60 (trace) and 0·42 (solvent *B*); L-xylose as acceptor, R_g 0·73 and 0·45 (solvent *B*), all of which gave pink stains with aniline oxalate.

A comparison of the suitability of other pentose sugars to act as glycosyl acceptors was then carried out by incubating D-lyxose, D-ribose, or L-arabinose (7 %, w/v) with cellobiose (7 %, w/v) and algal extract (0·7 %, w/v). A control cellobiose–D-xylose mixture was also prepared. In all cases, new sugars were formed which appeared to be glucosylpentose disaccharides. The R_X values of the products were: from D-lyxose 0·38 (solvent *B*), from D-ribose 0·44, from L-arabinose 0·28, and from xylose 0·43 and 0·27 (trace). With ribose and xylose, the new sugar could be detected within 24 hr.; with lyxose and arabinose, 8 and 15 days' incubation respectively were required.

Control digests showed that oligosaccharide

synthesis did not occur from glucose or xylose alone.

The greatest trans-β-glucosylation occurred with xylose, and this reaction was therefore examined on a larger scale.

Enzymic synthesis of glucosylxylose. Cellobiose (10·0 g.), D-xylose (10·0 g.) and *C. rupestris* fraction (0·3 g.) in water (40 ml.) were incubated at 35° for 14 days. Paper-chromatographic analysis (solvent *B*) showed the presence of a satisfactory amount of the component with R_X 0·43. The digest was heated to inactivate the enzyme, concentrated under reduced pressure, and placed on a charcoal–Celite column (86 × 6 cm.). The glucose and xylose were eluted with water, and the disaccharides (cellobiose and a sugar shown below to be glucosylxylose) with increasing concentrations of aqueous ethanol (0 → 10 %). Approx. 770 mg. of the glucosylxylose was collected; this was freed from traces of glucose and cellobiose by chromatography on Whatman 3MM paper with solvent *B*, and crystallized from ethanol.

Characterization of glucosylxylose. The sugar was homogeneous by paper chromatography, with R_G 1·02 and R_X 0·64 (solvent *A*), and R_X 0·43 (solvent *B*), and gave a pink stain with aniline phthalate and *p*-anisidine; it had m.p. 120°, $[\alpha]_D - 6{\cdot}4°$ in water (*c*, 2·8) and the reducing power of a disaccharide (xylose standard).

Hydrolysis with 0·67N-sulphuric acid for 6·5 hr. at 100° gave, by Pridham's (1956) method, equimolar proportions of glucose and xylose. After oxidation of the disaccharide with hypoiodite, an acid hydrolysate contained glucose but not xylose. Incubation with emulsin at pH 5·0 and 35° gave glucose and xylose.

In view of the mode of synthesis of the sugar, and of the fact that D-xylose exists in solution in the pyranose form, the above preliminary experiments indicate that it is a β-D-glucopyranosyl-D-xylopyranose. The following evidence shows the presence of a 1:3-linkage:

(1) On heating with sodium acetate–phenylhydrazine hydrochloride, a crystalline osazone was formed; after two recrystallizations from ethyl acetate, the bright-yellow crystals had m.p. 205–206° (cf. m.p. 213–215° reported by Barker *et al.* 1957). An acid hydrolysate of the osazone contained glucose and not xylose. These findings eliminate the possibility of a 1:2-linkage.

(2) On oxidation with sodium metaperiodate in the dark at room temperature, a rapid initial reduction of 3·02 mol.prop. was observed. After 22 hr. this had increased to only 3·07 mol.prop. The theoretical values for a 1:3- and 1:4-linked disaccharide are 3·00 and 4·00 mol.prop., respectively. Under alkaline conditions, the sugar was over-oxidized by periodate, since on prolonged oxidation more than 7 mol.prop. of periodate was reduced. This behaviour is typical of oligosaccharides which give rise to intermediates containing malonaldehyde groups (cf. Hough & Perry, 1956).

(3) Confirmation of a 1:3-linkage was provided by a comparison of R_X values (solvents *B* and *C*) with those of an authentic sample of 3-*O*-β-D-glucopyranosyl-D-xylopyranose kindly given by Dr S. A. Barker (cf. Barker *et al.* 1957).

Ulva lactuca *extract*

A digest containing cellobiose (16 %, w/v) and algal extract (1 %, w/v) was incubated at pH 5·0. The presence of laminaribiose, gentiobiose and cellotriose was detected by paper chromatography within 24 hr. Similar results were obtained after 3, 5 and 7 days' incubation. In contrast, a 1 % solution of cellobiose was completely hydrolysed. During the hydrolysis of cellodextrin (1·7 %, w/v), traces of laminaribiose were detected during the first 3 days; after this time, only cellosaccharides were present.

Attempts to demonstrate trans-β-glucosylation from cellobiose to D-galactose, D-mannose, phenol or methanol were unsuccessful. Digests with cellobiose–acceptor mixtures did not contain new sugars (other than those obtained with cellobiose alone) or glucosides after incubation for periods up to 16 days; the phenol and methanol partly inhibited the enzymic reaction.

DISCUSSION

Recent investigations have shown that cellobiase preparations from various higher plants (e.g. sweet almonds, gentian roots, barley) and moulds (e.g. *Myrothrecium verrucaria*, *Aspergillus niger*, *A. aureus*, *A. flavus*) catalyse the formation of oligosaccharides from concentrated solutions of cellobiose (Crook & Stone, 1957; Anderson & Manners, 1959). The present work shows that marine algae contain similar enzyme systems. The tentative identification of laminaribiose, gentiobiose and cellotriose suggests that, with cellobiose as β-glucosyl donor, both glucose (OH at C-3 and C-6) and cellobiose may function as acceptor substrates. By contrast, D-galactose, D-mannose, L-sorbose, *N*-acetyl-D-glucosamine, phenol and methanol do not appear to satisfy the specificity requirements. In a similar experiment with *A. oryzae* β-glucosidases, Jermyn & Thomas (1953) were able to demonstrate the transfer to methanol of β-glucosyl residues from *p*-nitrophenyl β-D-glucoside, but not from cellobiose.

The synthesis of 3-*O*-β-D-glucopyranosyl-D-xylopyranose from a cellobiose–xylose mixture is of interest since D-xylopyranose has, with the

exception of C-5, the same configuration as D-glucopyranose. The formation of this disaccharide thus parallels that of laminaribiose. This behaviour is similar to that reported by Barker *et al.* (1957) for *A. niger* (152) and differs from that of another strain of this mould (E 19) which was unable to effect appreciable trans-β-glucosylation to D-xylose (Crook & Stone, 1957). It is presumed that the hydroxyl groups at C-2 and C-4 of D-xylose, and at C-2, C-3 and C-4 in the other pentoses are less favourable acceptor sites for the algal cellobiase.

D-Xylose is not an acceptor substrate for trans-α-glucosylation by extracts of *C. rupestris* (Duncan & Manners, 1958).

SUMMARY

1. Extracts of *Cladophora rupestris* and *Ulva lactuca* catalyse the synthesis of oligosaccharides from concentrated solutions of cellobiose. Laminaribiose, gentiobiose and cellotriose have been identified by paper chromatography.

2. On incubation of *C. rupestris* extract with cellobiose–pentose mixtures, glucosylpentose disaccharides are produced. 3-*O*-β-D-Glucopyranosyl-D-xylopyranose has been isolated from a cellobiose–xylose mixture and characterized.

3. The acceptor specificity of cellobiases from various biological sources is discussed.

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Metal Ions as Precipitants for Nucleic Acids and their Use in the Isolation of Polynucleotides from Leaves

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Many genetical, virological and metabolic studies will require better methods for isolating nucleic acids than are yet available. It is necessary to aim at complete extraction of the polynucleotides from the tissues, since it cannot be assumed that even a large fraction is representative of the whole; and the product should be obtained as nearly as possible in its native state, uncontaminated by other cell polymers. In the procedure to be described an attempt is made to fulfil these requirements. It is assumed that the principal variables affecting the solubility of polynucleotides are polynucleotide concentration, temperature, hydrogen-ion concentration, ionic composition, ionic concentration and dielectric constant of the medium.

It has been found that a high proportion of the nucleic acid extracted from leaves in 1·7 M-sodium chloride at 100° is precipitated when the solution is kept at 0°. Williams (1945) found that comparable extracts from plants made with 2·1 M-sodium chloride precipitated the extracted nucleic acid at pH values from 0·4 to 3·4 when kept at 2–5°, maximum precipitation occurring at pH 0·4–1·6. Holden & Pirie (1955*a*) obtained fractions of nucleic acids from leaves and yeast by precipitation from extracts by sodium chloride at pH 3·0. Crestfield, Smith & Allen (1955) used this salt at pH 7·0 to precipitate nucleic acid from extracts of yeast in an attempt to prepare undegraded yeast ribonucleic acid. No detailed study of this type of method has been published, nor has there been a systematic study of the interaction of multivalent cations with nucleic acids, although La^{3+}, Cu^{2+} and some other multivalent metal ions are known to be good polynucleotide precipitants. A study of certain details of these reactions has been made, primarily as a guide to the

perfection of isolation procedures. The close analogy with proteins which it has revealed shows the possibility of a much wider application of metal precipitations to the chemical manipulation of polynucleotide solutions.

METHODS

Dry weights. Nucleic acid solutions were freeze-dried and then heated in an oven at 105° overnight.

Total phosphorus. The method of Allen (1940), as modified by C. S. Hanes (unpublished work), was used for the estimation of phosphorus in nucleic acids and nucleic acid hydrolysates.

Nitrogen. This was estimated by the micro-Kjeldahl method.

Protein. An approximate value for the upper limit of protein in nucleic acid preparations was obtained by Osborne's method (Coghill, 1931), with the quantities recommended by Holden & Pirie (1955*a*). A sample of serum albumin (Armour's Fraction 5) was used as a standard.

Nucleotides and purine bases. The purine bases and pyrimidine nucleotides from ribonucleic acid were determined after hydrolysis for 1 hr. at 100° in N-HCl and chromatography in solvent A, made by taking 700 ml. of 2-methylpropan-2-ol at 26°, adding 132 ml. of constant-boiling HCl and making to 1 l. at 20° (Smith & Markham, 1950). Nucleotides were estimated after alkaline hydrolysis and chromatography in solvent B [propan-2-ol–water (7:3, v/v; Markham & Smith, 1952), with which NH_3 is present in the vapour phase] and also in solvent C [sat. $(NH_4)_2SO_4$–water–propan-2-ol (79:19:2, by vol.; Markham & Smith, 1951)]. The nucleotides were also detected and estimated by electrophoresis on Whatman no. 3 MM paper in the apparatus of Markham & Smith (1952).

Nucleotides and purine bases were detected on the papers by the photographic method of Markham & Smith (1949, 1951) and by the direct observation of fluorescence quenching (Holiday & Johnson, 1949). This required the addition of a disk 2 mm. thick of Chance glass filter OX 7 to the light-filter system of Markham & Smith (1949, 1951). For guanine and guanylic acid the fluorescence printing method of Smith & Markham (1950), see also Matthews (1954), was also used.

Deoxyribonucleic acid. This was detected and estimated by a modification (Burton, 1956) of the Dische diphenylamine reaction.

Buffers. Sørensen buffers were prepared according to Britton (1955). Other buffers used were: 0·05 M-potassium phthalate–HCl (pH 3–4); 0·05 M-potassium phthalate–NaOH (pH 4–6); 0·05 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris) maleate (pH 6–8); 0·05 M-boric acid (in 0·05 M-KCl)–NaOH (pH 8–10).

Nucleic acid preparations

The proportion of three different preparations precipitated by metal ions was measured under standard conditions.

Preparation 1. This was obtained by dissolving commercial yeast nucleic acid in a small volume of water and dialysing at pH 7·0 for 48 hr. with repeated changes of water. The dialysed solution was stored at −10°. The phosphorus-containing component of this material was mainly polyribonucleotide containing the usual bases, since 97% of the total P of samples hydrolysed for 1 hr. at 100° with N-HCl was accounted for in terms of the purine bases and pyrimidine nucleotides separated by chromatography with solvent A.

Preparation 2. Highly polymerized thymus nucleic acid was obtained by a slight modification of the method of Hammarsten (1924) and was kindly presented by Dr K. Bailey, F.R.S.

Preparation 3. Nucleic acid from the foliage of lucerne, *Medicago sativa*, was chosen as a representative of the nucleic acid preparations from leaves with which this work is concerned. The methods of preparation differed essentially only in the method of precipitation of the polynucleotide from the crude plant extract. The best general method for destroying hydrolytic enzymes in the higher plants is treatment of the coarsely chopped tissues with boiling aqueous ethanol. Holden & Pirie (1955*b*) found that a ribonuclease obtained from leaves differs from the better-known pancreatic ribonuclease in being comparatively heat-labile, but as no detailed study of the stability of leaf nucleases has been made the method of inactivation employed here is arbitrary.

Freshly picked tips (279 g.) of young growing shoots of *Medicago sativa* were washed in tap water and 30 g. batches chopped and dropped into 300 ml. of boiling aqueous 80% ethanol. The boiling was continued for 2 min. and the leaves were disintegrated in fresh, cold, aqueous 80% ethanol for 5 min. in a high-speed macerator. The fine suspension was filtered through a Büchner funnel prepared with a thin layer of kieselguhr. The pad was washed with aqueous 80% ethanol until the filtrate ran colourless, then with acetone until it again ran colourless. The pad was sucked dry and the dried leaf powder was extracted successively with 50, 30 and 30 ml. of Sørensen 0·04 M-phosphate buffer, pH 7·0 at 100°. Each extract was squeezed out through muslin and the three extracts were combined and filtered through kieselguhr. The solution was adjusted to pH 3·0 with dilute HCl and NaCl (10 g. for each 100 ml.) was dissolved in it. The nucleic acid precipitated slowly and after being kept overnight at 0° it was removed by centrifuging and dissolved in the minimum volume of Sørensen 0·06 M-phosphate buffer, pH 7·0. The nucleic acid was precipitated with 3 vol. of ethanol and a few drops of saturated $MgSO_4$ solution and kept overnight at −10° to ensure complete precipitation.

The precipitate was removed by centrifuging, dissolved in water and reprecipitated three times with ethanol. The aqueous solution of the third precipitate was centrifuged to remove insoluble matter and dialysed against distilled water with stirring at room temp. for 3 hr. and then for 24 hr. at 0° without stirring. The dialysed solution was made up to 200 ml., centrifuged at 0° for 30 min. at 20 000 *g* to remove the remaining traces of suspended matter and stored at −10°. The solution contained 0·9 g. of solid material [N, 14·1%; P, 7·1%; ϵ(P) 8950; ϵ(P) after alkaline hydrolysis, 11 300] of which 4·5% was deoxyribonucleic acid (DNA) and about 5% protein. A sample hydrolysed for 48 hr. at room temp. with N-KOH was brought to pH 1·0 with $HClO_4$ and the DNA was removed by centrifuging at 20 000 *g* for 30 min.; the pH was brought to 4·0 with KOH, insoluble $KClO_4$ was removed and samples of the nucleotide solution were evaporated to dryness; the purine nucleotides were hydrolysed to the free bases by heating at 100° with N-HCl for 1 hr. Chromatography in solvent A, measurement of the absorption spectra of the components at

pH 1·0, 7·0 and 12·0 and phosphorus determinations showed that 95% of the total P of the ribonucleic acid (RNA) fraction could be accounted for in terms of components very similar to, if not identical with, the known products of RNA hydrolysis.

RESULTS

Precipitation of nucleic acids with the chlorides of alkali metals and ammonium chloride

Most of the samples of nucleic acid were obtained from leaf powders, prepared by methods similar to those used in preparation 3, by extraction with unbuffered 1·7 M-NaCl at 100°. When diluted ten times with CO_2-free deionized water these extracts had a pH of about 6·3. The undiluted extracts contained about 0·4 mg. of polynucleotide/ml., which precipitated slowly at 0° until about 80% of it had come out of solution. However, contrary to expectation, no nucleic acid was precipitated when equal volumes of a diluted solution of preparation 3, containing 1·16 mg. of dry matter/ml., of 0·05 M-tris maleate buffer, pH 7·0, and 6·9 M-NaCl, or 3·9 M-KCl or 5·7 M-NH_4Cl were mixed at 0°, but up to 70% of the polynucleotide was precipitated by LiCl under similar conditions (Fig. 1).

The effect of pH on the precipitation of nucleic acid (preparation 3) by LiCl is shown in Fig. 2. The same effect of pH was obtained with a higher final concentration of LiCl when the P in the precipitate was measured. When the 1·64 M-LiCl was replaced by 2·3 M-NaCl, 1·3 M-KCl and 1·9 M-NH_4Cl there was no precipitation of nucleic acid from preparation 3 buffered between pH 3·0 and 10·0.

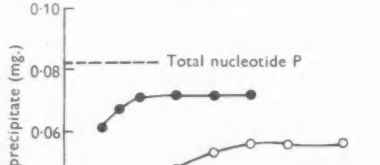

Fig. 1. Relation between the precipitation of leaf nucleic acid (diluted preparation 3) and the molarity of LiCl. ○, Nucleic acid solution (1 ml. containing 1·16 mg. of dry matter, i.e. 0·0825 mg. of P), 0·05 M-tris buffer, pH 7·0 (1 ml.), and LiCl solution (1 ml.) were mixed and the solution was kept at 0° overnight. ●, The same mixtures kept at 0° for 2 hr., then mixed with dioxan (1 ml.) and the solution kept at 0° overnight.

The addition of dioxan, acetone, ethanol or methanol greatly increased the precipitation of nucleic acid from preparation 3 by the above-mentioned salts. To 1 ml. of a diluted solution of preparation 3 containing 1·16 mg. of dry matter/ml. were added 1 ml. of buffer and 1 ml. of salt solution (9·0 M-LiCl, 6·9 M-NaCl, 3·9 M KCl or 5·7 M-NH_4Cl). These solutions were kept at 0° for 2 hr. before the addition of 1 ml. of dioxan and then were kept overnight at −5°. About 90% of the nucleic acid was precipitated by LiCl, NaCl and KCl at pH 3·0 and 8·0 and 75–80% at intermediate pH values. NH_4Cl caused precipitation of about 70% of the nucleic acid from pH 3·0 to 5·0 and much less at

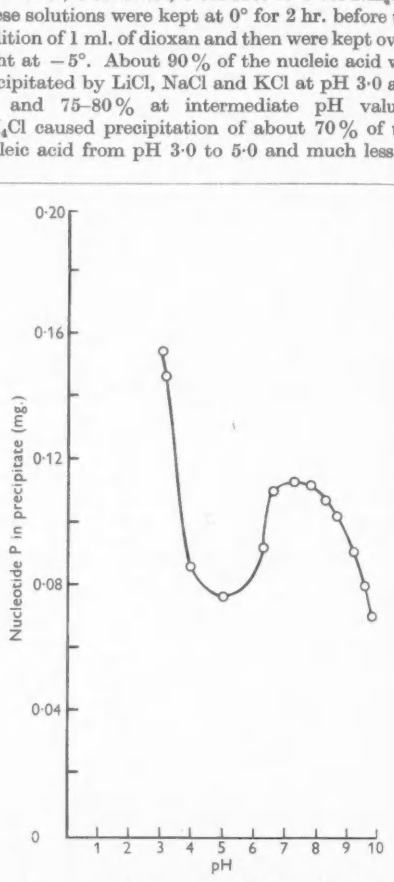

Fig. 2. Relation between the precipitation of leaf nucleic acid (diluted preparation 3) by 1·64 M-LiCl and the pH of the solution. A portion (2·5 ml.) of aqueous solution of diluted preparation 3, containing 1·16 mg. of dry matter/ml., was mixed with 2 ml. of buffer of chosen pH and 1 ml. of 9 M-LiCl was added. The mixture was kept at −5° overnight and the nucleic acid precipitate dissolved in Sørensen 0·033 M-phosphate buffer, pH 6·8. Buffers whose components absorb ultraviolet light were removed by precipitating the nucleic acid twice from water by the addition of 3 vol. of ethanol and a few drops of saturated $MgSO_4$; the nucleic acid was determined spectrophotometrically by measuring the extinction at 230 and 257 mμ.

higher pH values. With LiCl at pH 7·0 complete precipitation could not be achieved by increasing the salt concentration (see Fig. 1), but this was obtained with 3 M-LiCl by increasing the volume of added dioxan to 1·33 vol. Precipitations of preparations 1, 2 and 3 at pH 3·0 and 7·0 are recorded in Table 1.

Precipitation of nucleic acids with multivalent cations

Precipitation occurred with all multivalent metal ions at salt concentrations much lower than those required with alkali metal ions and it was possible to work with dilute ionic solutions and degraded nucleic acid preparation 1. The results are recorded in Table 2. The relative effectiveness of Al^{3+}, Co^{2+} and Ba^{2+} ions was the same with preparations 2 and 3 as with preparation 1 (Table 2), but the proportion of nucleic acid precipitated was increased in all cases.

Table 1. *Effect of the addition of dioxan on the precipitation of nucleic acids by 3 M-lithium chloride*

Nucleic acid solution (1 ml. containing 0·0825 mg. of nucleotide P), buffer [0·05 M-phthalate, pH 3·0, or 0·05 M-tris, pH 7·0 (1 ml.)] and 9 M-LiCl (1 ml.) were mixed and the solution was kept at 0° overnight before collection of the precipitate. Dioxan (1 ml.) was added 2 hr. after the solution had been made.

Nucleic acid preparation no.	Nucleic acid precipitated (%)			
	pH 3·0		pH 7·0	
	Without dioxan	With dioxan	Without dioxan	With dioxan
1	28	38	0	36
2	0	79	0	81
3	93	95	67	86

Table 2. *Precipitation of nucleic acid of preparation* 1

Nucleate solution (1 ml. containing 0·0825 mg. of P), 1 ml. of buffer (glycine–HCl for pH 3·0 and 0·05 M-tris maleate for pH 7·0) and 1 ml. of 0·01 M-cation solution were mixed and kept at room temp. for 1 hr. The precipitate was separated by centrifuging and the total P of the pellet was determined. In some experiments ethanol (1 ml.) was added 1 hr. after the addition of cation solution. The precipitate was collected at least 1 hr. later. The results with Pb^{2+} at pH 3·0 were obtained with unbuffered solutions adjusted with acetic acid.

Cation	Nucleic acid precipitated (%)			
	Without ethanol		With ethanol	
	pH 3·0	pH 7·0	pH 3·0	pH 7·0
La^{3+}	91	85	—	—
Al^{3+}	82	—	—	—
UO_2^{2+}	95	0	—	0
Fe^{3+}	83	0	—	0
Pb^{2+}	82	0	—	0
Fe^{2+}	58	—	75	—
Co^{2+}	27	0	67	0
Ni^{2+}	36	0	67	0
Mn^{2+}	38	0	72	0
Cu^{2+}	53	0	73	0
Ca^{2+}	0	0	65	0
Ba^{2+}	0	0	68	0
Zn^{2+}	0	0	69	0
Hg^{2+}	0	0	61	0
Mg^{2+}	0	0	49	0

Isolation of nucleic acid from leaf extracts by salt precipitation

Leaf powders were prepared as described for preparation 3. A minor modification, introduced in later preparations, was to precede the acetone extraction by extraction with warm ethanol–ether (3:1, v/v), which increases the extraction of lipoid substances. Method 1 (below) gives erratic results unless the following modification is made. After maceration in aqueous 80% ethanol the solid leaf material is removed by centrifuging for 20 min. and washed twice by resuspending in 80% ethanol and centrifuging for 20 min. It is then transferred to a Büchner filter and washed with acetone. In the methods described below the extractions of the powder with Sørensen 0·04 M-phosphate buffer, pH 7·0, at 100° were replaced by four extractions with unbuffered 1·7 M-NaCl at 100°.

Method 1: *precipitation by* 1·7 M-NaCl. The combined, filtered 1·7 M-NaCl extracts were kept at 0° for at least 24 hr. The nucleic acid precipitate was removed by centrifuging and extracted four times with borate buffer, pH 9·7, and the combined extract was adjusted to pH 4·0. Ethanol (3 vol.) and a few drops of saturated $MgCl_2$ solution were then added. The precipitation of nucleic acid was repeated twice and the third precipitate was dissolved in a small volume of water, brought to pH 7·0 and then dialysed against distilled water with stirring at room temp. for 3 hr., then at 0° for 4 days with daily changes of water. The dialysed solution was made to a known volume (usually 50–100 ml./300 g. fresh wt. of leaves) and stored for analysis. During storage some non-nucleotide material was precipitated and was removed before analysis by centrifuging at 0° for 1 hr. at 20 000 *g*.

Method 2: *precipitation with* Al^{3+} *ions in* 0·57 M-NaCl. The combined 1·7 M-NaCl extract from the leaf powder was filtered through kieselguhr and mixed with 1 vol. of glycine buffer, pH 3·0 (glycine–NaCl–HCl; all 0·1 M) and 1 vol. of 0·1 M-$Al_2(SO_4)_3$. The mixture was kept overnight at 0° and the precipitated polynucleotide component was removed by centrifuging at 0°. The precipitate was dissolved in 0·05 M-tris maleate buffer, pH 7·0, and the nucleate reprecipitated by the addition of ethanol (3 vol.). This precipitation was repeated twice with the addition of a few drops of saturated NaCl solution. The third precipitate was dissolved in water and insoluble matter was removed by centrifuging for 30 min. at 0° and 20 000 *g*.

Method 1 suffers from the disadvantage of giving incomplete precipitation and the product appears to be inferior to that obtained by method 2, which gives the better product by the analytical criteria with virtually complete precipitation of the polynucleotide from leaf extracts.

The preparations were stored at −10° as nucleates in water. These solutions contained 1–4 mg. of nucleate/ml. and appear to undergo no major degradation during storage, since their ϵ(P) value remained unchanged for at least

Table 3. *Analyses of nucleic acid preparations from leaves*

Source	Method	Fresh wt. of leaves (g.)	Dry wt. of leaves (g.)	Nucleic acid isolated (mg.)	Percentage of total nucleic acid*	Light absorption (mμ) min.	Light absorption (mμ) max.	Analyses of isolated nucleic acid: N (%)	P (%)	N/P	ε(P)	ε(P) after alkali	Increase of ε(P) (%)	DNA (%)	Protein (%)
Beta maritima. Young leaves	1	264·2	24·2	164·5	82	231	257·5	15·3	8·6	3·92	8 000	11 100	39	4·0	<2
B. vulgaris, strain KDNB. Young leaves	1	307·5	27·1	159·4	78	231	258	14·7	8·6	3·79	8 000	11 700	46	4·0	<2
B. vulgaris, strain T49/147. Polyploid. Young leaves	1	307·3	28·0	168·0	77	230·5	258	15·4	7·65	4·45	7 950	11 000	39	4·2	<2
B. vulgaris, strain Battle's. Young leaves	1	326·0	25·7	141·5	76	230	257·5	15·0	8·4	3·93	7 600	11 100	46	4·3	<2
B. vulgaris, strain Battle's infected with beet yellows virus. Young leaves	1	317·4	31·0	201·5	85	230	257·5	16·7	7·2	5·15	7 550	10 900	44	5·1	2
B. vulgaris 1. Very young leaves	2	30	—	20·1	>98	231	259	12·4	7·3	3·76	7 750	10 800	39	5·2	<3
B. vulgaris 2.	2	30	—	35·4	>98	231	259	13·3	7·6	3·88	—	—	—	3·7	<2
Kale 1. Very young leaves	2	30	—	52·8	>98	230	257·5	13·8	8·0	3·82	7 800	9 400	21	1·9	<1
Kale 2. Very young leaves	2	30	—	45·9	>98	230·5	257·5	13·2	7·5	3·91	7 920	10 900	38	2·4	1
B. vulgaris. Very young leaves	2†	60	—	78·4	>98	231	257·5	12·2	8·05	3·35	8 750	9 100	4	2·5	<1

* Values obtained from the absorption curves at 257 mμ of suitably diluted solutions of the precipitated nucleic acid and unprecipitated nucleic acid. Interference by non-polynucleotide material in the solution of unprecipitated nucleic acid was minimized by precipitation with ethanol and centrifuging as described in the text. Where necessary an allowance was made for unspecific general absorption.

† La^{3+} ions substituted for Al^{3+} ions.

Fig. 3. (*a*) Absorption curves of leaf extracts: 103 ml. of 1·7 M-NaCl extract from 30 g. fresh wt. of beet leaves. ○, solution diluted 1:3 [100 ml. was precipitated with $Al_2(SO_4)_3$ by method 2 and nucleic acid removed (see Fig. 3*b*)]. □, Supernatant solution (300 ml., pH 3·0) before and after centrifuging at 20 000 *g* for 30 min. ●, Supernatant solution at pH 12·0; 50 ml. of supernatant was precipitated with 3 vol. of ethanol. Δ, Precipitate redissolved in 50 ml. of water; ▲, the same at pH 12·0. (*b*) Absorption curve of nucleate of Fig. 3(*a*) in 300 ml. of water.

1·5 years. The degree of purity, the proportion of RNA and DNA, the degree of polymerization and the yield of the products of these two methods may be judged from Table 3. A third method was similar to method 1 except that after the addition of NaCl 0·4 vol. of dioxan was added. Since the yield was not appreciably increased and the products had a comparatively low contents of N and P (N, 6–8%; P, about 4%) the method was abandoned.

Until much more is known about nucleic acids and their function the relative merits of different methods of preparation cannot be evaluated. However, as shown by the curves in Figs. 3*a* and 3*b*, method 2 has the advantage of completeness and this was confirmed by using Al^{3+} ions to precipitate a further fraction of polynucleotide from the five preparations by method 1 shown in Table 3. In this method, after the separation of nucleic acid from the NaCl extract of leaves a portion of both the RNA and the DNA remained in solution. After centrifuging, the supernatant fluids from the five preparations were reprecipitated four times with 3 vols. of ethanol, dissolved in 50 ml. of water and treated with Al^{3+} ions according to method 2. The nucleic acid precipitate was removed by centrifuging at 20 000 *g* for 1 hr.

Many preparations have been made by methods 1 and 2, and the supernatant liquids, after the removal of the nucleic acid precipitate, were tested spectrophotometrically for the presence of polynucleotide. Method 1 never precipitated the whole of the polynucleotide and method 2 never left a detectable quantity of it in solution. The material employed in all cases was immature foliage, usually young leaves of sugar beet, and it is improbable that differences in leaf material were responsible for the observed differences in precipitation by the different methods. A direct comparison of the two methods on three separate samples of leaf material showed that method 1 precipitated 74, 71 and 76% of the amount of nucleic acid precipitated by method 2.

Completeness of separation of nucleic acids from leaves

Leaf residues were extracted from 1 to 2 days at room temperature with 2N-KOH. The extracts were neutralized with 60% $HClO_4$ and, after concentration, chromatographed in solvents B and C and submitted to electrophoresis on paper in ammonium formate buffer, pH 3·5, to separate the ribonucleotides. The DNA, which remained unhydrolysed, was precipitated with ethanol and measured colorimetrically by Burton's (1956) method, which permitted the easy detection of DNA in concentrations down to 20 μg./ml. The limits of detection by the other methods are shown in Table 4. The results for DNA and electrophoresis of ribonucleotides in extracts of the residue from 30 g. fresh weight of leaves showed that the amounts of both types of components, if present, were beyond the limits of the tests, i.e. the residue from the 30 g. of fresh leaves contained less than 0·16 mg. of RNA and less than 0·1 mg. of DNA. These quantities are respectively less than 1·6 and 1·0% of the total nucleic acid usually isolated from a 30 g. sample of young leaves. Since some components of the leaf residues ran in positions overlapping those normally occupied by the ribonucleotides the chromatographic methods were unsuitable for this material.

DISCUSSION

Under conditions which avoid the known dangers of degradation, 97% of the nucleic acid has been extracted from sugar-beet leaves. It was separated from the extracts by salts in proportions ranging from approximately 75% to at least 98% of the total polynucleotide, depending on the salt used. It is still impossible to determine the extent to which such preparations are artifacts and the relative values of the methods depend very largely upon the use for which the preparations are intended and the nature of the plant material from

Table 4. *Smallest quantities of purines and ribonucleotides detected after chromatography and electrophoresis*

Material was applied in bands approx. 6 × 1·5 cm. to Whatman no. 3 paper for chromatography and to Whatman no. 3 MM paper for electrophoresis. Results are expressed in μg. detected per band.

Substance	Chromatography			Electrophoresis at pH 3·5 in ammonium formate buffer
	Solvent A	Solvent B	Solvent C	
Adenine	4–6	—	—	—
Guanine	4–6 (0·5–1)*	—	—	—
Adenylic acid	—	4–10	36	4
Guanylic acid	—	4–10 (4)*	40 (4)*	4–8 (4)*
Cytidylic acid	—	3–10	25	3–6
Uridylic acid	—	3–10	30	3–6

* Limit when the bands were detected by fluorescence printing.

which the extracts are made. The methods yield products containing a high proportion of polynucleotide which compare favourably with good preparations of ribonucleic acid from sources other than higher plants. The ε (P) values are low and increase by about 40 % on alkaline hydrolysis, which, by currently accepted standards, is an indication that the preparations are not highly degraded.

The interactions of metal ions with proteins have been studied in the light of modern concepts, but there have been no comparable studies of their reactions with nucleic acids. However, the work of Smith & Alberty (1956) and others has shown the existence of ionic complexes between metals and such substances as adenosine di- and tri-phosphate. The work of Hammarsten (1924), Hammarsten, Hammarsten & Teorell (1928), Chargaff, Vischer, Doniger, Green & Misani (1949) and Caspersson, Hammarsten & Hammarsten (1935) suggests that DNA forms complexes with Fe^{3+}, La^{3+} and Cu^{2+} ions; Stern & Steinberg (1953) studied compounds of this type formed by DNA and several of the ions of rare-earth metals. Davidson & Waymouth (1944) showed that a similar reaction occurs between RNA and La^{3+} ions. Metal-sequestering properties of nucleic acids were demonstrated by Neuberg & Roberts (1949) and the present work demonstrates the high affinity of nucleic acids for some metal ions.

Among biological polymers the polynucleotide molecule has the largest number and variety of potential electron-pair donors available for complex formation, and the best explanation of the results in this paper would be that complexes of the predominantly ionic type are formed between the polynucleotides and most, if not all, of the true metals. The metal ions may be divided into three main groups according to their ability to precipitate polynucleotides from aqueous solutions. Alkali-metal ions and NH_4^+ ions are the weakest precipitants and require about 2 M-concentrations of salt to cause appreciable precipitation. This type of precipitation seems to be a salting-out phenomenon. The alkaline-earth metal ions and Zn^{2+}, Hg^{2+} and Mg^{2+} (all elements in group II of the Periodic Table) occupy an intermediate position and the ions of the transitional elements, lead and tervalent metals such as Al^{3+} and La^{3+} are the most powerful precipitants. In this respect the metal ions fall into the same groups as when they are classified according to their ability to form chelate compounds with inorganic polyphosphates in which the combining groups of the ligand are the phosphoryl oxygen and the free acidic groups of the adjacent phosphate groups in the chain. Van Wazer & Campanella (1950) found that alkali metals and similar ions form weak complexes with inorganic polyphosphates; the other metals form stronger complexes of which the most stable are those formed by UO_2^{2+} ions and the transitional elements. The parallel suggests that nucleic acids may form similar chelate complexes. Whatever the explanation of the observations which have been described, a thorough investigation of the interaction of polynucleotides with metal ions and with organic solvents appears to be necessary.

SUMMARY

1. The precipitation of nucleic acids by metal ions has been studied.

2. Two methods have been devised for the isolation of nucleic acids from leaves, based on precipitation by metal ions.

3. Analytical results on ten preparations from leaves by these methods are given. These suggest that relatively pure, undegraded polynucleotides are produced.

4. Unextracted nucleic acid in leaf residues could not be detected.

5. The mechanism of the interaction between metal ions and nucleic acids is discussed.

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Bacterial Degradation of the Nitrobenzoic Acids

2. REDUCTION OF THE NITRO GROUP*

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Reduction of the aryl nitro group by animal tissues (Beuding & Jolliffe, 1946; Parker, 1952), moulds (Zucker & Nason, 1955) and bacteria (Smith & Worrel, 1950; Egami, Ebata & Sato, 1951; Yamashina, 1954; Saz & Slie, 1954*a*, *b*) has been observed. The fundamental importance of this reaction is emphasized by the work of de la Haba (1950) and McElroy & Spencer (1956), from which the suggestion arose that nitro compounds are probably intermediates in the reduction of nitrates by green plants and micro-organisms, and the finding that moulds and higher plants, in the absence of added nitro compounds or their presumed precursors, elaborate enzymes capable of metabolizing nitro compounds (Bush, Touster & Brockman, 1951; Shimoda, 1951; Raistrick & Stössl, 1958; Little, 1957).

The appearance of arylamines in cultures of *Nocardia* species growing on nitrobenzoic acids was noted by Cain (1958*a*), but Cartwright & Cain (1959) found that these compounds appeared to have little significance in the oxidative breakdown of *p*- and *m*-nitrobenzoates, although this is less certain with the *ortho*-isomer.

The study of the reduction of nitrobenzoates by *Nocardia* species and a strain of *Pseudomonas fluorescens* described in this paper includes investigations with cell-free extracts and the stimulation of a nitroreductase by flavin adenine dinucleotide, an effect which had previously been observed only in *Escherichia coli* among the bacteria (Saz & Martinez, 1956).

EXPERIMENTAL

Organism

The organisms used were strains of *Nocardia erythropolis*, *N. opaca*, *Nocardia* M 1 and *Pseudomonas fluorescens* isolated by enrichment methods and grown in bulk on a chemically defined medium by the techniques described by Cain (1958*a*). The procedures for obtaining adapted cells, washed suspensions and cell-free extracts were those described by Cartwright & Cain (1959). The particular strain of organisms used is named in the description of each experiment. Absence of contamination was checked by examination of stained smears and plating of samples.

Chromatography

The solvent systems used were (all quantities by vol.): (A) butanol–acetic acid–water (4:1:5); (B) ethanol–aq. NH_3 soln. (sp.gr. 0·880)–water (40:1:6). Arylamino compounds were detected by Ehrlich's reagent or by diazotization and reaction with *N*-naphthylethylenediamine. Phenolic compounds were detected by exposure to ultraviolet light, spraying with $FeCl_3$ or with an alkaline 1% solution of diazotized I.C.I. 5091 (4-aminophenyl-2′-diethylaminoethylsulphone; L. Light and Co. Ltd.).

Incubations

Unless otherwise stated, incubations were performed in Warburg flasks (about 16 ml. volume) attached to manometers which were left open to air. Temperature was maintained at 30° during shaking at 120 strokes/min.

Estimations

Arylamines. The procedure was essentially that of Bratton & Marshall (1939) as modified by Glazko, Wolf & Dill (1949). Calibration curves were prepared by the use of known compounds. Under the conditions used, the coloured complexes of *o*, *m*- and *p*-aminobenzoic acid showed molar-extinction coefficients of 7700, 10 960 and 12 510, respectively. The aminobenzoic acids have linear responses over the range 0–5 μg./ml. (10 μg./ml. with the *ortho*-isomer) and 0·3 μg./ml. of each could be accurately estimated. Anthranilic acid had a lower extinction with consequent less accurate analytical results at low concentrations. For very small quantities of *p*-aminobenzoic acid (less than 0·05 μg./ml.) a microbiological assay was used.

Protein. This was estimated either by the Folin method of Lowry, Rosebrough, Farr & Randall (1951) or by the biuret method of Gornall, Bardawill & David (1949).

Growth. The growth of the *Nocardia* species was estimated turbidimetrically at 530 mμ with the precautions mentioned by Cain (1958*a*).

Ultraviolet spectra. These were plotted from readings on a Unicam SP 500 instrument.

Microbiological assay

Medium. Cutts & Rainbow (1950) described a yeast, *Saccharomyces cerevisiae* 47, having an absolute requirement for *p*-aminobenzoic acid in a synthetic medium supplemented with other growth factors. A culture of this yeast was used to identify *p*-aminobenzoic acid in *p*-nitrobenzoate

* Part 1: Cartwright & Cain (1959).

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cultures by measuring, turbidimetrically, growth of the yeast on a chemically defined medium supplemented with either authentic *p*-aminobenzoic acid or the filtered culture fluid produced by growth of *N. erythropolis* on *p*-nitrobenzoic acid. The medium for yeast growth, which was adjusted to pH 5·0–5·2 and sterilized by autoclaving at 15 lb./in.^{2} for 15 min., contained (in 1 l.): glucose, 40 g.; $(NH_4)_2HPO_4$, 4 g.; KH_2PO_4, 0·2 g.; $MgSO_4,7H_2O$, 0·1 g.; $CaCl_2,6H_2O$, 0·25 g.; lactic acid, 3 g.; 50% (w/v) potassium lactate solution, 12 ml.; trace element solution (Barnett & Ingram, 1955), 10 ml. The vitamin supplement, sterilized by filtration, was added aseptically to the cooled medium to give the following final concentrations (μg./l.): *meso*-inositol, 10 000; (+)-biotin, 2; calcium pantothenate, 1000; thiamine HCl, 1000; pyridoxine hydrochloride, 1000; nicotinic acid, 1000; riboflavin, 50; *p*-aminobenzoic acid, 100.

Assay procedure. The *p*-aminobenzoic acid requirement of yeast 47 was replaced by L-histidine (10 mg./ml.), DL-methionine (20 mg./ml.) and adenine (10 mg./ml.) (Dr C. Rainbow, personal communication), and the sensitivity of yeast 47 to *p*-aminobenzoic acid was markedly increased if the inocula for new cultures were grown for two or three subcultures on a medium devoid of *p*-aminobenzoic acid but supplemented with these factors.

The arylamine produced by incubation of washed cells of *Nocardia erythropolis* with *p*-nitrobenzoic acid was assayed for *p*-aminobenzoic acid after removal of the cells by centrifuging. Samples of this supernatant, together with standards of authentic *p*-aminobenzoic acid of the same concentration (as measured colorimetrically), were sterilized by filtration through a sintered-glass filter (pore size 0·95 μ) before addition to tubes of the previously sterilized medium. The final volume in each tube was 5 ml. and quadruplicate sets of unknown, standard and control tubes were inoculated with 0·05 ml. of a washed suspension of yeast 47. The inoculum culture was grown for 3 days on the *p*-aminobenzoic acid-deficient medium supplemented with adenine, methionine and histidine. It was harvested and washed aseptically, and resuspended to 5 ml. in sterile water. The inoculated test cultures were incubated at 30° for 3 days and the extinction was then read on a Unicam SP. 350 D.G. spectrophotometer at 445 mμ in 10 mm. light-path glass cells.

Chemicals

Most chemicals were commercial products which were twice recrystallized in this Laboratory. *p*-Hydroxylaminobenzoic acid was synthesized by the method of Bauer & Rosenthal (1944), and repeatedly recrystallized till free of *p*-aminobenzoic acid as revealed by chromatography. It did not melt below 300°. *p*-Nitrosobenzoic acid was prepared and purified by the method of Cartwright & Cain (1959), the product agreeing in properties with those given for this isomer by Alway (1904); 2-hydroxy-4-nitrobenzoic acid (*p*-nitrosalicylic acid) and *p*-aminosalicylic acid were synthesized by the method of McGhie, Morton, Reynolds & Spence (1949), 3-hydroxy-4-nitrobenzoic acid by the method of Brenans & Prost (1924) and the corresponding amino compounds by methods essentially the same as those used to reduce *p*-nitrosalicylic acid. Oxidized and reduced di- and tri-phosphopyridine nucleotides (DPN, DPNH, TPN) were obtained from L. Light and Co. Ltd. and were 95% pure on spectrophotometric assay. Flavinadenine dinucleotide (FAD) (80%) and riboflavin 5-phosphate (FMN) were products of the Sigma Chemical Co., St Louis, Mo., U.S.A.) Adenosine di- and tri-phosphate (ADP and ATP, as the sodium salts) were not assayed but were the products as obtained from L. Light and Co. Ltd.

Buffers

Sørensen (Na_2HPO_4–KH_2PO_4) phosphate buffers and 2-amino-2-hydroxymethylpropane-1:3-diol (tris)-HCl buffers were used. Concentrations used are mentioned in the text.

RESULTS

Arylamine production by washed suspensions of Nocardia erythropolis *CA* 4 *and* Pseudomonas fluorescens *NC* 3

A correlation between the *p*-nitrobenzoic acid added to the medium and the maximum amount of arylamine produced by *N. erythropolis* in growing cultures was first observed by Cain (1958*a*). Washed suspensions of this organism and *P. fluorescens* also reduced *p*-nitrobenzoic acid, and *N. opaca* and *Nocardia* M1 reduced *o*- and *m*-nitrobenzoic acid respectively under similar conditions.

The ability to form arylamine from *p*-nitrobenzoic acid was examined with suspensions of *N. erythropolis* grown on *p*-nitrobenzoic acid (adapted) or on

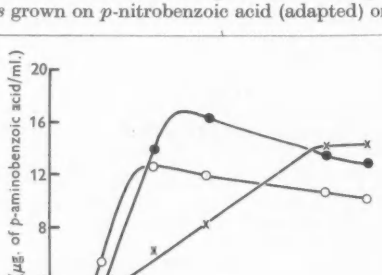

Fig. 1. Arylamine production by washed suspensions of *Nocardia erythropolis* and *Pseudomonas fluorescens*. The incubation mixtures contained: sodium *p*-nitrobenzoate, 1 m-mole; 0·067 M-phosphate buffer, pH 7·4, 200 ml.; cell suspension (*N. erythropolis*, 600 mg. dry wt.; *P. fluorescens*, 450 mg. dry wt.). Final volume, 250 ml. Adapted cells were harvested from defined medium + *p*-nitrobenzoate after growth for 6 days; unadapted cells were harvested from glucose + $(NH_4)_2SO_4$ medium solidified with agar. Portions (3 ml.) were removed at intervals, the reaction was stopped by the addition of 0·5 ml. of 10% (w/v) trichloroacetic acid and estimated for arylamine. Incubations were performed at 30° in 1 l. Erlenmeyer flasks loosely plugged with cotton wool and agitated vigorously on a Microid flask shaker (approx. 300 oscillations/min.; 5 cm. throw). *p*-Aminobenzoic acid: 1 μg./ml. is equivalent to 7·25 μm-moles/ml. ●, *N. erythropolis*, adapted cells; ×, *N. erythropolis*, unadapted cells; ○, *P. fluorescens*, adapted cells.

glucose and $(NH_4)_2SO_4$ (unadapted) and also with suspensions of *P. fluorescens* grown on *p*-nitrobenzoic acid. When incubated with *p*-nitrobenzoic acid in phosphate buffer adapted cells of both organisms reduced the nitro group, with a maximum level of arylamine appearing in 8–10 hr. and then decreasing slowly (Fig. 1). With unadapted cells, the rate of arylamine production was slower, but the maximum level eventually reached was comparable with that from adapted cells. There was no appreciable increase in cell mass or any contamination over this period of time. Suspensions of non-viable dried cells of *N. erythropolis*, grown on *p*-nitrobenzoic acid, also performed this reduction and, in addition, reduced 3-hydroxy-4-nitrobenzoic acid to the corresponding amino compound. Similar preparations of *N. opaca* grown with *o*-nitrobenzoic acid reduced 3-hydroxy-2-nitrobenzoic acid to 3-hydroxyanthranilic acid.

Identification of p-*aminobenzoic acid*

Spectrophotometric identification. At pH 3·5, *p*-aminobenzoic acid has a sharp peak at 225 mμ, which coincides with a minimum in the *p*-nitrobenzoic spectrum. Cell-free filtrates from 7-day growing cultures and from incubations of washed cell suspensions with *p*-nitrobenzoic acid and buffer, when diluted and adjusted to pH 3·5, also showed a sharp peak at 225 mμ, suggesting that *p*-aminobenzoic acid was present in significant quantities. This was confirmed by its isolation from such filtrates.

Chromatographic identification. The acidified supernatants from cultures and from all suspensions similar to those described above were extracted continuously with ether for 30–40 hr.; samples of the concentrated extracts were applied to papers together with authentic *p*-aminobenzoic acid and run in the solvent mixtures A and B. In each solvent system spots moving with the same R_F and yielding the same colour reactions as authentic *p*-aminobenzoic acid were obtained. The naturally produced arylamine failed to separate from authentic material when both were applied in admixture for chromatography.

Microbiological identification. The usefulness of the microbiological method for identifying *p*-aminobenzoic acid in cultures of *N. erythropolis* grown with *p*-nitrobenzoic acid as the substrate was dependent upon the lack of any inhibitory effect against yeast 47 of *p*-nitrobenzoic acid (or other metabolic products) remaining in such cultures. Results obtained on the chemically defined medium with the vitamin supplement containing authentic *p*-aminobenzoic acid (100 μg./l.) showed that *p*-nitrobenzoic acid, up to 0·02 M, produced no diminution of growth. Our inositol sample (from vegetable sources), a possible source of extraneous *p*-aminobenzoic acid, was also shown microbiologically to contain no *p*-aminobenzoic acid. Table 1 demonstrates the identity with *p*-aminobenzoic acid of the arylamine produced by *N. erythropolis* from *p*-nitrobenzoic acid.

Chemical isolation of p-*aminobenzoic acid.* This was undertaken to give final proof of the identity of the arylamine. A filtered *p*-nitrobenzoic acid culture medium (8 l.) after growth of *N. erythropolis*, which showed by colorimetric analysis a total arylamine content of 65 mg., was evaporated *in vacuo* to 300 ml. and, after acidification and removal of the precipitate by filtration, the volume was further reduced to 50 ml. Addition of ethanol (100 ml.) precipitated much inorganic material, which was filtered off. Further evaporation to 25 ml. was followed by acidification with dil. HCl and two extractions with ether to remove the bulk of the reddish-brown pigment. The aqueous layer was neutralized with dil. NaOH and propan-2-ol added to 80 % (v/v) to precipitate most of the NaCl. The filtrate was concentrated to about 20 ml., acetic acid (2 ml.) added and the solution continuously extracted with ether for 4 days or until the arylamine content of the aqueous phase was minimal. After evaporation of the ether, the residue was dissolved in water (5 ml.) and applied to a column (1 cm. × 15 cm.) of Amberlite IR-120 (H) resin (Chromatography Grade), which was then washed with water (200 ml.) and 1 mN-acetic acid (50 ml.), whereupon some contaminating brown pigment was eluted. The *p*-aminobenzoic acid fraction was eluted with 100–150 ml. of 2 N-acetic acid. The acidic eluate was extracted continuously with ether for 40 hr., evaporated to dryness *in vacuo* to remove acetic acid and the ether-soluble residual material recrystallized (charcoal) from water (2 ml.) to give fawn-coloured needles (12 mg., m.p. 174–175°). This substance did not depress the m.p. of authentic *p*-aminobenzoic acid; it supported the growth of

Table 1. *Activity of the arylamine formed by reduction of* p-*nitrobenzoic acid for the* p-*aminobenzoate-requiring yeast* 47

Quadruplicate tubes containing the *p*-aminobenzoate-deficient basal medium with the additions shown were inoculated with yeast 47 and incubated for 64 hr. at 30°. Details are given in the Experimental section.

Additions to medium	Final concn. in tubes (μg./ml.)	Growth (E 445 mμ) Mean	Range
p-Nitrobenzoic acid	16·70	0·011	0·006–0·015
p-Aminobenzoic acid	0·027	0·630	0·620–0·640
Arylamine* from supernatant	0·027	0·624	0·618–0·638
None	—	0·018	0·011–0·026

* Estimated colorimetrically as *p*-aminobenzoic acid.

yeast 47 and behaved identically with *p*-aminobenzoic acid on chromatograms. Further proof of the identity of the material was obtained from conversion into the *N*-acetyl derivative by treating with excess of acetic anhydride and sodium acetate on a steam bath for 15 min. The product, isolated by dilution with water and extraction into ether, crystallized from water to form white needles, m.p. 247–248°, undepressed on admixture with *N*-acetyl-*p*-aminobenzoic acid. (All melting points are uncorrected.)

Identification of anthranilic acid

Anthranilic acid was identified from cultures of *N. opaca* grown on *o*-nitrobenzoate (*a*) spectrophotometrically by its absorption max. at 310 mμ, pH 7·0, (*b*) chromatographically by R_F and its violet–blue fluorescence under u.v. light and (*c*) by isolation on ion-exchange columns by procedures essentially similar to those described for *p*-aminobenzoic acid. Fewer experiments were done with *m*-nitrobenzoic acid, and the arylamine produced in cultures grown on this substrate was considered by analogy to be *m*-aminobenzoic acid.

Removal of p-*aminobenzoic acid by washed suspensions*

The disappearance of *p*-aminobenzoic acid was studied by incubating suspensions of adapted and unadapted cells of *N. erythropolis* with *p*-aminobenzoic acid in phosphate buffer, pH 7·0, under sterile conditions with shaking at 30°. Since this experiment extended over 48 hr. sterile precautions were observed when samples were withdrawn at intervals, analysed for *p*-aminobenzoic acid and examined chromatographically for hydroxy compounds. The marked difference in the behaviour of the two types of cells is shown in Fig. 2. Failure of cells grown with *p*-nitrobenzoic acid to metabolize *p*-aminobenzoic acid immediately provides further evidence that this acid is not on the direct pathway of *p*-nitrobenzoic acid degradation, although the ultimate metabolites of both *p*-nitrobenzoic acid and *p*-aminobenzoic acid may be the same. No hydroxy compounds were detected but this may be due to their further metabolism in phosphate buffer (cf. Cartwright & Cain, 1959).

Reduction with cell-free extracts

Cell-free extracts of *N. erythropolis* and *N. opaca* were no longer able to oxidize the *para*- and *ortho*-isomers respectively of nitrobenzoic acid but still brought about reduction of these substrates although their activity was much less than that of the intact cells. Nevertheless, the reduction occurring was sufficient to enable investigations of the process to be made.

Fig. 2. Disappearance of *p*-aminobenzoic acid on incubation with washed suspensions of *Nocardia erythropolis*. Adapted and unadapted cells were grown as described in Fig. 1 and harvested under aseptic conditions. The organisms were incubated at 30° with the aeration conditions described in Fig. 1 in sterilized incubation mixtures (100 ml. final volume) containing: *p*-aminobenzoic acid, 10 μmoles; 0·067 M-phosphate buffer, pH 7·2; initial dry wt. of adapted cells was 90 mg. and of unadapted cells was 93·2 mg. Portions (3 ml.) were taken with sterile precautions, deproteinized with 10% trichloroacetic acid (0·5 ml.) and estimated for residual *p*-aminobenzoic acid (1 μg./ml. is equivalent to 7·25 μm-moles/ml.). ●, Adapted cells; ○, unadapted cells.

Table 2. *Reduction of* p-*nitro-*, p-*nitroso- and* p-*hydroxylamino-benzoic acid by extracts of* Nocardia erythropolis *CA* 2

Each flask contained: substrate, 5 μmoles; $MgSO_4$, 10 μmoles; DPN, 0·4 μmole; L-cysteine, 4 μmoles; 0·1 M-phosphate buffer, pH 7·0, 2 ml.; extract, 4·4 mg. of protein (Expt. 1), 2·8 mg. of protein (Expt. 2); water to 4 ml. Since *p*-hydroxylaminobenzoic acid gives a colour reaction with the Bratton–Marshall test (Bauer & Rosenthal, 1944), control flasks containing (*a*) substrate + boiled enzyme, or (*b*) no substrate and active enzyme, to correct for extraneous colour and endogenous arylamine respectively were included. After deproteinizing with 1 ml. of 10% (w/v) trichloroacetic acid the contents were analysed for *p*-aminobenzoic acid. The reaction proceeds at constant rate, therefore results are expressed as total arylamine formed/hr./mg. of protein extract. Expt. 1 was performed with extracts from actively growing cells (log phase) and Expt. 2 with extracts from 6-day-old cells (stationary phase).

Substrate	*p*-Aminobenzoic acid produced (μm-moles/hr./mg. of protein) Expt. 1	Expt. 2
p-Nitrobenzoic acid	6·2	3·4
p-Nitrosobenzoic acid	7·0	4·5
p-Hydroxylaminobenzoic acid	6·4	3·1
None	0·2	0·1

Stepwise reduction of the nitro group. *p*-Hydroxylaminobenzoic acid and *p*-nitrosobenzoic acid are likely intermediates between *p*-nitrobenzoic acid and *p*-aminobenzoic acid. Cell-free extracts of *N. erythropolis* grown with *p*-nitrobenzoic acid were prepared by ultrasonic disintegration, under the conditions described by Cartwright & Cain (1959), and incubated with *p*-nitrobenzoic acid and *p*-hydroxylaminobenzoic acid under the conditions given in Table 2. In these experiments the correction of Glazko *et al.* (1949) for extraneous colour obtained in the controls with boiled extract was applied in estimating the content of *p*-aminobenzoic acid. The results agree with reduction proceeding via nitroso and hydroxylamino compounds, the rates of reduction of these compounds being comparable with or higher than that of *p*-nitrobenzoic acid itself.

Production of p-*nitrosobenzoic acid and* p-*hydroxylaminobenzoic acid from* p-*nitrobenzoic acid.* The role of nitroso- and hydroxylamino-benzoic acid as intermediates in the reduction process was demonstrated by the action of extracts of *N. erythropolis* on *p*-nitrobenzoic acid followed by chromatography of the reaction mixtures. *p*-Aminobenzoic acid and hydroxylaminobenzoic acid were visualized by direct spraying with Ehrlich's reagent; nitrosobenzoic and *p*-nitrobenzoic acid were reduced by an acidic 0·5% $SnCl_2$ spray before using Ehrlich's reagent. The nitroso compound, which moved close to *p*-nitrobenzoic acid, was more clearly distinguished by spotting at 5 mm. intervals on a separate sheet with microdroplets of a solution of diphenylamine in conc. H_2SO_4, which produced a bright-red colour with nitrosobenzoic acid but had no effect on the other metabolites. Table 3 shows the progressive reduction of *p*-nitrobenzoic acid recorded chromatographically.

Some properties of the reductase complex. The reductive capacity of cell-free extracts of both *N. opaca* and *N. erythropolis* prepared by ultrasonic disintegration was lower than that of an equivalent quantity of washed cells. However, the extent of reduction in the absence of added H-donors was dependent on the amount of extract present and more evidence for the enzymic nature of the activity was provided by the observation of only very slight arylamine production with a boiled extract. The optimum pH for the reduction of *p*-nitrobenzoic acid was 7·0 in both phosphate and tris buffer, but the rate of reduction as well as the total yield of arylamine was greater in the presence of phosphate. Centrifuging an extract at 104 000 *g* for 20 min. produced a particle fraction with negligible activity after washing, but the supernatant soluble portion remained active. Of several thiols tried, only L-cysteine had a stimulatory effect on the reductase activity, but was not essential (cf. Saz & Slie, 1954*b*). Other reducing agents, including ascorbic acid, did not affect the reaction in any way.

The amounts of arylamine produced by extracts of *N. erythropolis* grown on glucose or *p*-nitrobenzoic acid, corrected for protein concentration, were approximately the same, this finding being explicable on the grounds that reduction was not due to a specific enzyme but was a result of the dehydrogenase activity of other enzymes for which the nitro group acts as H-acceptor (Nason, 1956). Support for this concept was obtained from a study of the effect of extracts on other nitro compounds (Table 4), from which it appears that, whereas

Table 3. *Progressive reduction of* p-*nitrobenzoic acid by* Nocardia *extracts*

To the main compartment of each of four Thunberg tubes, each fitted with a side arm, was added: sodium *p*-nitrobenzoate, 20 μmoles; FAD, 2 μmoles; $MgSO_4$, 10 μmoles; DPNH, 40 μmoles; 0·05 M-tris buffer, pH 7·2, 7 ml. The hollow cap contained cell-free extract, 20·4 mg. of protein; the side arm contained 1 ml. of 10% (w/v) trichloroacetic acid. A control tube contained boiled enzyme. The tubes were evacuated and refilled with O_2-free N_2 three times, the contents of cap and main compartment were mixed and incubation was carried out at 30° with gentle agitation. Tubes were removed at 0, 2, 5 and 10 hr.; the reaction was stopped by tipping in the trichloroacetic acid before opening the tubes, and the protein precipitate removed by centrifuging. The supernatants were extracted with cold ether (3 × 10 ml.) and solvent was removed under reduced pressure; the residue was taken up in 0·5 ml. of ethanol. Portions (0·1 ml.) were applied, together with authentic markers, to Whatman no. 542 paper and the chromatograms run overnight in solvent mixture B. Intensity of spots were estimated visually as −, ±, +, + + etc.

	Compounds present			
Time (hr.)	*p*-Nitrobenzoic acid	*p*-Nitrosobenzoic acid	*p*-Hydroxylamino-benzoic acid	*p*-Aminobenzoic acid
0	+ + + +	−	−	−
2	+ + +	+	±	±
5	+	+ +	+	+ + +
10	±	+	±	+ + + +
10 (boiled enzyme)	+ + + +	±	−	±

highest activity was associated with substrates containing both the carboxyl and nitro group, the activity was markedly non-specific.

Cofactors in nitro reduction. Considerable reduction of nitrobenzoate was achieved in the absence of added H-donors, indicating a high level of endogenous H-donors and the dehydrogenase system(s) making these available. Dialysis of the supernatant fraction from extracts centrifuged at 104 000 *g* for 20 min. against distilled water produced a progressive fall in reductase activity to a level as low as 25 % of the original after 48 hr. at 0–4°. The activity was completely restored by the addition of undialysed boiled extract and to the extent of 70–80 % by DPN in catalytic quantities; TPN was ineffective with extracts of both species. It is apparent therefore that dialysis of this duration has little effect on the endogenous H-donor and that the loss of activity is the result of removal of the carrier, DPN. The effects of the addition of these and other possible cofactors are shown in Table 5. There was a linear relationship between recovery of activity and added DPN with 48 hr. dialysed extracts up to a concentration of 1 mM-DPN.

Table 4. *Non-specific nitroreductase activity of extracts of* Nocardia erythropolis *CA* 4

Each flask contained: substrate (as shown); L-cysteine, 4 μmoles; 0·05 M-tris buffer, pH 7·2, 2 ml.; extract, 4·1 mg. of protein; water to 4 ml. Temp. 30°; incubation time, 10 hr.; aerobic conditions. Arylamine was estimated as *p*-aminobenzoic acid after deproteinization with 10 % (w/v) trichloroacetic acid, 0·5 ml. The organism had been grown upon *p*-nitrobenzoic acid.

Substrate	Amount of substrate (μmoles)	Arylamine formed (μm-moles/ml.)
m-Dinitrobenzene	Saturated solution	50
2:4-Dinitrobenzoic acid	10	91
Nitrobenzene*	10	12
2:4-Dinitrophenol	10	5
2:5-Dinitrophenol	10	10
'Nitrofurazone'	5	4
Chloramphenicol†	3	6
2-Fluoro-4-nitrobenzoic acid	10	62
o-Nitrobenzoic acid‡	10	7
m-Nitrobenzoic acid‡	10	29
p-Nitrobenzoic acid	10	31
3-Hydroxy-4-nitrobenzoic acid	5	34

* Prepared as a stable emulsion by ultrasonic irradiation.
† Pure crystalline compound supplied by Parke Davis and Co. Ltd.
‡ Estimated as the *o*- and *m*-aminobenzoate.

Table 5. *Stimulation of activity by added cofactors*

Each flask contained: *p*-nitrobenzoic acid, 5 μmoles; 0·05 M-tris buffer, pH 7·2, 2 ml.; extract (dialysed for 84 hr.), 4·3 mg. of protein; cofactors to final concentration shown; water to 4 ml. Temp. 30°; incubation time, 17 hr.; aerobic conditions.

Addition	Concn. of addition (μmoles/flask)	*p*-Aminobenzoic acid (μm-moles/ml.)
None	—	21
Boiled undialysed extract	0·4 ml./flask	50
DPN	0·4	42
Adenosine triphosphate	20·0	12
Adenosine diphosphate	20·0	12
Adenosine diphosphate + K_2HPO_4	20·0 40·0	30
TPN	0·4	18
TPN	4·0	21
Undialysed extract used as enzyme (no additions)	—	40

Nocardia extracts dialysed for 80–96 hr. (or more) required the addition of a dibasic acid in addition to DPN for the restoration of full activity. L-Malate, fumarate, succinate and aspartate were the most effective. These acids and DPN apparently act by maintaining the supply of DPNH, since DPNH in substrate quantities under both aerobic and anaerobic conditions replaced the stimulation of nitroreductase by an acid + DPN (Table 6). Malic dehydrogenase, fumarase and succinic dehydrogenase activities have all been observed in these extracts (unpublished results) and aspartase has previously been found in *N. erythropolis* (Cain, 1958*a*). The acids most likely act therefore by conversion into malate, which maintains DPN in the reduced form through malic dehydrogenase activity. Cytochrome *c* (0·1 mM) did not influence the stimulatory effect of succinate on dialysed extracts.

The stimulatory effect of flavins on arylamine production by extracts of *Nocardia* species dialysed against water was first observed with a boiled pig-heart preparation with 48 hr.-dialysed extracts, suggesting that flavins are involved in H-transport. This effect was also examined with FAD and FMN. Fig. 3 shows that only FAD stimulated arylamine production by *N. erythropolis* extracts, which had been dialysed against an acid solution for 72 hr. to effect a more rigorous removal of endogenous flavin. Extracts of *N. opaca* responded to FMN to some extent, but riboflavin was without effect on extracts of both species. It was observed that TPN would not substitute for DPN in flavin-enriched extracts and that flavins had little effect on fresh crude extracts.

Effect of inhibitors. Both hydroxylamine and nitrite acted as inhibitors of nitroreductase activity, the effect of the former being comparable in magnitude with that of *p*-chloromercuribenzoic acid (Table 7). By analogy with their effects upon nitrite and hydroxylamine reductase (Taniguchi, Sato & Egami, 1956), hydroxylamine probably affects the conversion of nitroso- into hydroxylamino-benzoate and nitrite the second stage, i.e. hydroxylamino- to amino-benzoate; chromato-

Table 6. *Requirements of an extensively dialysed extract for a reduced diphosphopyridine nucleotide source*

Each flask contained: *p*-nitrobenzoic acid, 5 μmoles; L-cysteine, 4 μmoles; $MgSO_4$, 10 μmoles; 96 hr.-dialysed extract, 7·4 mg. of protein; 0·1 M-phosphate buffer, pH 7·0, 2 ml. Additions (or water) as shown; total volume 4·5 ml. Temp. 30°; incubation time, 14 hr.; aerobic conditions.

Additions	Concn. of addition (μmole/ml.)	DPN added (μmole/ml.)	Arylamine formed (μm-moles/ml.)
None	—	—	18
None	—	0·1	29
Boiled undialysed extract	0·4 ml./flask	—	34
L-Malate	1	0·1	37
DL-Aspartate	1	0·1	33
Fumarate	1	0·1	43
Succinate	1	0·1	38
L-Malate + DPNH	1 + 1	0·1	39
DPNH	1	—	38
DPNH (boiled enzyme)	1	—	7

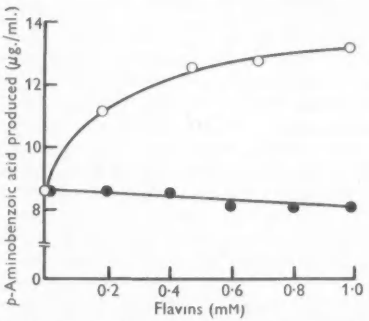

Fig. 3. Stimulation of *Nocardia erythropolis* nitroreductase by flavins. Each flask contained: *p*-nitrobenzoic acid, 5 μmoles; DPN, 4 μmoles; L-cysteine, 4 μmoles; extract (dialysed for 72 hr. against frequent changes of water made to pH 5·5 with N-acetic acid), 5·25 mg. of protein; flavins were added as shown; water to 4 ml. The reaction mixtures were made up in diffuse light, the bath was covered with black cloth and the incubation performed overnight in the dark. Incubation time, 12 hr.; temp. 30°. *p*-Aminobenzoic acid: 1 μg./ml. is equivalent to 7·25 μm-moles/ml. ○, FAD; ●, FMN.

Table 7. *Inhibition of reduction of* p-*nitrobenzoate*

Each Warburg flask contained: *p*-nitrobenzoic acid, 5 μmoles; extract, 4·3 mg. of protein; 0·05 M-tris buffer, pH 7·2, 2·5 ml.; inhibitor to concentration shown; L-cysteine, 4·5 μmoles; water to 4·5 ml. Temp. 30°; incubation time, 17 hr. Inhibition is expressed as the percentage decrease in arylamine of that produced in absence of inhibitor.

Addition	Concn. of addition (mM)	Inhibition (%)
Hydroxylamine	10	86·3
	5	95·3
	0·1	50·0
	0·01	2·0
Sodium nitrite	5	32·5
	1	16·0
	0·1	1·5
	0·01	0
p-Chloromercuribenzoic acid	1	80·0
	0·01	7·5

graphy of incubation mixtures inhibited with either hydroxylamine or nitrite showed the presence of *p*-nitrosobenzoic acid. The effect of hydroxylamine shown in the Table is quite different from that demonstrated in oxidative metabolism of *p*-nitrobenzoic acid (Cartwright & Cain, 1959), where nitrite was probably split off and concentrations of hydroxylamine as low as 0·1 mM were completely inhibitory.

Metal requirement in nitro group reduction. Separate samples of freshly prepared extracts of *N. erythropolis* were dialysed against KCN, 1:10-phenanthroline and ethylenediaminetetra-acetic acid (EDTA) (all mM) for 48 hr. at 4°. There was a marked fall in activity with these agents, and almost complete inhibition with phenanthroline; KCN was the least effective, causing 35–40% inhibition. Table 8 shows the reversal of this inhibition by addition of metal ions to extracts prepared from actively growing and stationary-phase cells. Mg^{2+}, Mn^{2+} and Fe^{2+} ions are particularly effective out of several dibasic ions tested, a result which has been repeatedly confirmed for different cell batches over a period of 2 years.

Growth experiments in media rendered metal-free by the methods of Waring & Werkman (1943) showed that, with *N. erythropolis*, Mn^{2+} ion, involved in the *Escherichia coli* nitroreductase (Saz & Slie, 1954*a*), was without effect on either growth or arylamine production in the presence of excess of Fe^{2+} ions (1 μg./ml.), but increase in Fe^{2+} ions from zero to 0·02 μg./ml., in the presence of 0·1 μg. of Mn^{2+}/ml., increased the arylamine production in cultures 40-fold but growth was increased only 13-fold, whereas increase in Fe^{2+} from zero to

Table 8. *Reversal of the effects of dialysis against chelating agents by added metal ions*

Each flask contained: *p*-nitrobenzoic acid, 5 μmoles; L-cysteine, 4 μmoles; DPN, 0·4 μmole; added metal ion, 4 μmoles; 0·1 M-phosphate buffer, pH 7·0, 1·5 ml.; dialysed extract, 3·3 mg. of protein (Expt. 1) and 2·8 mg. of protein (Expt. 2). Temp. 30°; incubation time, 18 hr.; aerobic conditions.

All metals were added as sulphates except $FeCl_3,6H_2O$ and Na_2MoO_4.

Expt. 1 was performed with extracts from 6-day-old (stationary-phase) cells and Expt. 2 with 2-day-old (log-phase) cells. The figures given represent the stimulation, which is expressed as the arylamine produced compared with control in the absence of metal (= 100).

	Chelating agent used in dialysis							
	KCN		1:10-Phenanthroline		EDTA		8-Hydroxyquinoline	
Added metal ion	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2
Fe^{2+}	—	100	325	357	182	129	106	—
Fe^{3+}	—	—	118	100	100	—	197*	—
Mg^{2+}	—	177	118	100	330	290	—	—
Mn^{2+}	—	100	120	171	180	224	110	—
MoO_4^{2-}	—	100	110	100	163	100	121	—

* There was significant reduction to Fe^{2+} ions in the presence of cysteine over the duration of the experiment.

0·04 μg./ml. increased arylamine 80-fold but growth only 50-fold.

Attempts to separate components of the enzyme complex. All efforts to separate the distinct enzymes responsible for the stages nitro to nitroso, nitroso to hydroxylamino and hydroxylamino to amino by standard techniques of protein purification failed completely. Only an overall concentration of the total reductase activity was achieved.

DISCUSSION

The experiments described establish the ability of growing cultures, washed cell suspensions and cell-free extracts of *Nocardia* species, and of washed cell suspensions of *P. fluorescens* NC 3, to reduce the nitro group of the nitrobenzoic acids, in particular of the *para*-isomer, to the amino group with the accumulation of the corresponding aminobenzoic acids, which, after reaching a maximum concentration, slowly disappear. The ultimate fate of the amino acids has not been fully established in the present study. A consideration of our findings, together with the evidence of oxidative metabolism (Cartwright & Cain, 1959), indicates that reductive dissimilation is a subsidiary process. *p*-Aminobenzoic acid does not lie on the direct oxidative pathway because no appreciable oxidation of this substrate has been observed with cells grown with *p*-nitrobenzoic acid; it is removed far more rapidly by cells grown with glucose than by cells grown with *p*-nitrobenzoic acid. In addition, *N. erythropolis* grows poorly on *p*-aminobenzoic acid as sole carbon, nitrogen and energy source compared with its growth on *p*-nitrobenzoic acid. This further confirms the difference between *p*-nitrobenzoate metabolism in *Nocardia* (Cartwright & Cain, 1959) and in *Pseudomonas*, in which Durham (1958) indicated that *p*-aminobenzoic acid was a direct intermediate.

Our previous findings suggest that direct oxidation of *p*-nitrobenzoic acid yields nitrite and *p*-hydroxybenzoic acid, which is oxidized further; reduction of the nitro group which occurs slowly at the same time results in the gradual accumulation of *p*-aminobenzoic acid, which eventually reaches a level sufficient to induce some *p*-aminobenzoic acid-metabolizing activity in the cells. The existence of a prolonged induction period before cells grown with *p*-nitrobenzoic acid will rapidly remove *p*-aminobenzoic acid, whereas cells grown on glucose do so immediately, could be explained by the existence of a common precursor of the *p*-nitrobenzoic acid and *p*-aminobenzoic acid-metabolizing enzymes which is almost completely removed during growth on *p*-nitrobenzoic acid. In any case, if *p*-nitrobenzoic acid metabolism proceeded directly through *p*-aminobenzoic acid, the release of the nitro group as nitrite would be ruled out. Evidence in support of the reduction of the nitro group not being a primary reaction is provided by the observations that *Nocardia* M1, which also produces arylamine, releases 70% of nitro nitrogen as nitrite, and that cultures of *N. opaca* and *N. erythropolis* grown, respectively, on *o*- and *p*-nitrobenzoic acid also yield nitrite in small amounts. The difference between the nitrite levels in *Nocardia* M1 and the two other species was explained by Cartwright & Cain (1959).

The reduction of the nitro group by *Nocardia* species probably proceeds via the corresponding nitroso and hydroxylamino compounds. The facts that both are reduced by extracts at similar rates to *p*-nitrobenzoic acid and that their presence has been demonstrated chromatographically as products of the action of extracts on *p*-nitrobenzoic acid itself is regarded as evidence of their role. Saz & Martinez (1956) have found that *p*-nitrosobenzoic acid appears in the reduction of *p*-nitrobenzoic acid

by a strain of *E. coli*, and Yamashina, Shikata & Egami (1954) presented chromatographic evidence for the formation of the corresponding *o*-nitroso- and hydroxylamino-benzoic acid from *o*-nitrobenzoic acid by extracts of *Bacillus pumilus*. This stepwise reduction of the nitro group to amino group is obviously of widespread occurrence, hydroxylamino compounds having also been demonstrated and isolated from animal tissues (Beuding & Jolliffe, 1946) and *Neurospora crassa* (Nason, 1956).

Although Hondo, Otsuka & Shimoda (1956) reported an apparently specific nitroreductase, most nitroreductase activity appears to be non-specific, being the result of known enzyme systems oxidizing their substrates and using the nitro group as an H-acceptor, e.g. succinic dehydrogenase (Westfall, 1943), alcohol and formic dehydrogenases (Greville & Stern, 1935) and yeast diaphorase (Brodie & Gots, 1952). The ability of extracts of any one of several *Nocardia* species to reduce a variety of nitro compounds, nitroso- and hydroxylamino-benzoic acid, and the additional property of an extract from an organism grown on one isomer of nitrobenzoic acid to reduce the other two (Cain, 1958*a*, and unpublished results), together with the failure to separate enzyme fractions capable of mediating in only one reaction of the chain, suggests that the *Nocardia* enzymes are likewise non-specific.

The reductase activity of these organisms has other properties in common with the enzymes previously found in moulds and bacteria. The nitroreductase of *E. coli* was DPN-mediated (Saz & Slie, 1954*b*), and like that of *N. erythropolis* had a flavin requirement (Saz & Martinez, 1956). An FAD-linked *m*-dinitrobenzene reductase in *Neurospora crassa* has been reported (Zucker & Nason, 1955). Several nitrite reductases responsible for reducing nitrogen at the same oxidation level as in nitro compounds are also flavin-linked; those from *Neurospora* (Nicholas & Nason, 1954) and *E. coli* (Nicholas & Nason, 1955) have both flavin and molybdenum requirements. This is worthy of note in view of the apparent identity of the chloramphenicol nitroreductase and nitrite reductase of *Streptococcus haemolyticus* (Egami *et al.* 1951) and the inhibition of *N. erythropolis* nitroreductase by hydroxylamine, which characteristically inhibits nitrite reductase (Taniguchi *et al.* 1956).

Yamashina (1954), Yamashina *et al.* (1954) and Villanueva (1959) have all noted the inhibitory effect of potassium cyanide and sodium azide on nitroreductases in bacteria; our results agree with this. Villanueva found that EDTA, 8-hydroxyquinoline and $\alpha\alpha'$-dipyridyl had but little effect on a nitroreductase from a *Nocardia* species but the first two compounds and especially 1:10-phenanthroline caused extensive inhibition in the nitroreductase from *N. erythropolis*. It is not clear from Villanueva's preliminary report whether the chelating agents were added to the reaction mixture or whether, as in our experiments, the extracts were dialysed against such agents. We have found that the latter method is the more rigorous. The differences may thus be merely qualitative. The addition of metal ions to crude extracts had no effect, but addition to extracts dialysed against chelating agents revealed the stimulation of nitroreductase given by Mg^{2+}, Mn^{2+} or Fe^{2+} ions. Fe^{2+} was the only metal effective in increasing arylamine in growth experiments.

The variations in metal-stimulation responses given by extracts dialysed against the different reagents is probably a result of the metal–chelate association constants. 1:10-Phenanthroline, for instance, is particularly effective for Fe^{2+} ions. Addition of Fe^{2+} ions to a phenanthroline-dialysed extract therefore results in a greater stimulation than would result from addition of other bivalent ions.

Other minor pathways of nitrobenzoate metabolism by reduction of the nitro group exist in these organisms. 3-Hydroxy-4-aminobenzoate is produced by *N. erythropolis* from *p*-nitrobenzoic acid, and *N. opaca* gives 3-hydroxyanthranilic acid from *o*-nitrobenzoic acid (Cain, 1958*b*). These compounds are also produced by the action of dried preparations of the organisms on the corresponding hydroxynitrobenzoic acids. The order of hydroxylation and reduction has not been deduced but the work of Partridge, Bonner & Yanofsky (1952) suggests that in micro-organisms there is no direct hydroxylation of anthranilic acid as observed in mammalian metabolism by Kotake & Shirai (1953).

SUMMARY

1. Growing cultures, washed cell suspensions and cell-free extracts of *Nocardia* species and a strain of *Pseudomonas fluorescens* reduce the nitrobenzoic acids to the corresponding aminobenzoic acids.

2. Reductive dissimilation is a subsidiary process, not a primary mode of attack on the nitro group, and the aminobenzoic acids do not lie on the direct oxidative pathway of nitrobenzoic acid metabolism.

3. Reduction of the nitro group to the amino level probably proceeds in *Nocardia erythropolis* via the corresponding nitroso- and hydroxylaminobenzoic acid.

4. No separation of the enzymes responsible for the individual steps in the reductive process was obtained with standard protein-fractionation techniques.

5. The nitroreductase is non-specific and requires reduced diphosphopyridine nucleotide as H-donor. Its activity is stimulated by flavinadenine dinucleotide and inhibited by hydroxylamine and nitrite.

6. Dialysis against ethylenediaminetetra-acetic acid, 1:10-phenanthroline, 8-hydroxyquinoline and potassium cyanide causes extensive inhibition and reveals the requirement for a bivalent metal. Mn^{2+}, Mg^{2+} and, in particular, Fe^{2+} ions are active. The effect of the last-named was also observed in experiments with metal-free media.

7. The significance in the overall metabolism of several hydroxynitrobenzoic acids isolated on chromatograms from experiments with *Nocardia* species could not be deduced.

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Studies on the Succinate–Neotetrazolium Reductase System

ACTIVATION BY VITAMIN K_3

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The formation of insoluble formazans by the enzymic reduction of tetrazolium salts has been extensively used in the histological demonstration of dehydrogenase activity. Recently several reports have been concerned with the application of this procedure to the quantitative estimation of dehydrogenase activity in tissue homogenates (Kun & Abood, 1949; Sprinz & Waldschmidt-Leitz, 1953; Zöllner & Rothemund, 1954; Shelton & Rice, 1957; Sourkes & Lagnado, 1957).

The mechanism of the reaction is still unknown, as is the point(s) at which tetrazolium couples with

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the respiratory chain. Attempts to elucidate this point have been made by Brodie & Gots (1951) and by Ling, Su & Tung (1957). In this paper the enzyme system coupling the oxidation of succinate with the reduction of neotetrazolium chloride is termed succinate–neotetrazolium reductase.

In attempts to use the procedure given by Shelton & Rice (1957) for routine assay of succinate–neotetrazolium reductase, it was found that the formazan produced did not increase proportionately with increasing amounts of tissue added; in the text this is called the non-linear response. This suggested either that the reagents of the experimental system had affected the enzyme or alternatively that there had been dilution of an essential cofactor. A cofactor requirement for succinate–neotetrazolium reductase has been indicated by the work of Bril (1954), Sprinz & Waldschmidt-Leitz (1953) and by Sugimura & Ono (1956). A similar non-linear response in tetrazolium reduction has been reported by Sourkes & Lagnado (1957) for monamine oxidation.

This paper describes preliminary work on the mechanism of the non-linear response and the stimulation of succinate–neotetrazolium reductase by vitamin K_3.

METHODS

The rats used were black-and-white adult females of the Medical Research Council strain; body wt. was 180–200 g. They were fed on a diet described by Parkes (1946). Mice were adult males (body wt. 35–40 g.) of the Tuck strain. Animals were killed by cervical dislocation and liver suspensions (1:10) were prepared in ice-cold aqueous 0·25 M-sucrose in a Potter–Elvehjem (1936) type of homogenizer with a plastic pestle (clearance 0·005 in.). For experiments with mitochondrial fractions the centrifuging scheme was as follows: the tissue suspension was spun at 600 *g* for 10 min. in a refrigerated M.S.E. Angle 13 centrifuge at 0° to remove the so-called cell debris and nuclei. This spinning was repeated on the supernatant. The supernatant from the second nuclear centrifuging was spun at 10 000 *g* for 10 min. to sediment the large-particle or mitochondrial fraction. This fraction was resuspended in the original volume of 0·25 M-sucrose. In all separations of sediment and supernatant a pipette with a bent tip was used. For experiments with mammary-gland suspensions, homogenization was carried out as described by Greenbaum & Slater (1957).

The following reagents were used: (Na_2HPO_4–NaH_2PO_4) buffer, 0·1 M, pH 7·4; disodium succinate (succinic acid was recrystallized and the pH adjusted to 7·4 with sodium hydroxide), 0·5 M, pH 7·4; disodium ethylenediaminetetraacetic acid (EDTA), 0·1 M, pH 7·4; neotetrazolium chloride, 1% (w/v) in water; 2-amino-2-hydroxymethylpropane-1·3-diol (tris, recrystallized), 0·1 M, adjusted to pH 7·4 with hydrochloric acid. Vitamin K_3 was added in suspension form prepared as follows: 20 mg. of vitamin K_3 was dissolved in 3 ml. of absolute ethanol; 12 ml. of 0·2% ox-plasma albumin in 0·1 M-Na_2HPO_4–NaH_2PO_4 buffer (pH 7·4) was then added and the resulting suspension was kept cold and protected from bright light. Other reagents are mentioned at the appropriate position in the text.

Except where otherwise indicated in the text, incubations were carried out aerobically at 37° with 0·8–1·0 ml. of stock solution of the following composition: 20 ml. of Na_2HPO_4–NaH_2PO_4 buffer, 2 ml. of sodium succinate and 1 ml. of EDTA. A sample of tissue suspension (0–0·2 ml.) was added to each tube, giving a final pre-incubation volume of 1 ml. The mixture was pre-incubated for (usually) 2 min., after which neotetrazolium chloride (1·5 mg.) was added to each tube and the resulting reaction was finally stopped with 1 ml. of 10% trichloroacetic acid solution. The formazan was extracted with 4 ml. of ethyl acetate and the extinction at 510 mμ was obtained with a Hilger Uvispek spectrophotometer. The incubation time and other additions to the incubation mixture are given in the text.

RESULTS

Preliminary experiments. The relationship between the formazan produced (extinction at 510 mμ) and the amount of tissue added was not linear with rat liver (Fig. 1, curve *a*). Similar results

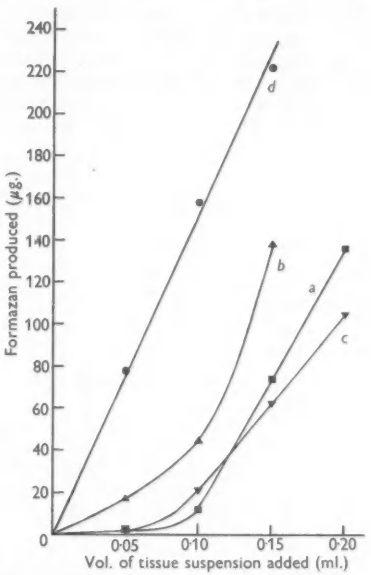

Fig. 1. Formazan production by various tissue suspensions. Assay conditions were as described in the Methods section; pre-incubation time was 2 min. ■, Rat-liver homogenate: 100 mg. (wet wt.) of tissue/ml., incubation time 15 min.; ▲, rat-mammary-gland homogenate: 200 mg. (wet wt.)/ml., incubation time 30 min.; ▼, mouse-liver homogenate: 100 mg. (wet wt.) of tissue/ml., incubation time 10 min.; ●, Keilin–Hartree-type heart-muscle preparation: 13·1 mg. of protein/ml., incubation time 3 min.

were obtained with rat-mammary-gland and mouse-liver suspensions (Fig. 1, curves *b* and *c*, respectively); linearity was obtained under identical conditions with a Keilin–Hartree-type heart-muscle preparation kindly provided by Dr M. B. Thorn (Fig. 1, curve *d*).

The non-linear response found with the above-mentioned types of tissue suspension was not affected by recrystallization of reagents, by varying the ionic strength of the phosphate buffer or by changing the buffer to tris. In attempts to obtain a linear response, tissue suspensions were prepared either in water or in 0·25M-sucrose and were then frozen and thawed five times. Such treatment did not lead to any appreciable increase in formazan production and did not induce linearity of response with increasing amounts of added tissue (Table 1). Homogenization in 0·5M-sucrose or in 0·25M-sucrose containing 0·1M-nicotinamide was also without effect on the non-linear response.

The overall production of formazan was increased by the addition of EDTA to the incubation medium, although EDTA did not affect the non-linear response (Table 1). The addition of 1 mg. or 20 mg. amounts of ox-plasma albumin did not alter the non-linear response (Table 1), although occasionally there was an increase in formazan production.

The addition of riboflavin 5′-phosphate (2 μmoles), lipoic acid (1 μmole) or formazan (0·02 μmole) did not affect the formation of formazan in the presence of fresh homogenate. Preincubation of fresh homogenate with riboflavin 5′-phosphate with or without adenosine triphosphate (ATP) for 20 min. before adding neotetrazolium chloride was without effect.

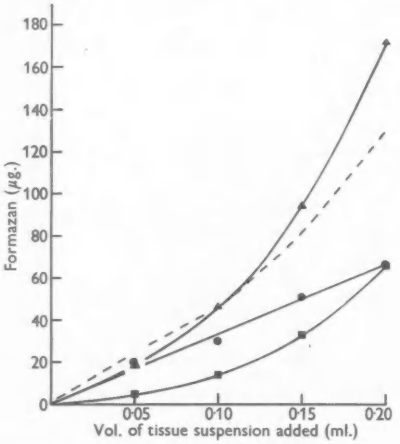

Fig. 2. Formazan production by rat-liver suspensions frozen and thawed three times. Substrates used were ■, succinate (50 μmoles); ●, DPN (2 μmoles) plus β-hydroxybutyrate (10 μmoles); ▲, succinate + DPN + β-hydroxybutyrate. Assay conditions were as described in the text; preincubation time was 2 min., incubation time 10 min. The broken curve is the arithmetical sum of curves —■— and —●—.

Effect of diphosphopyridine nucleotide. With tissue or mitochondrial suspensions, the addition of diphosphopyridine nucleotide (DPN) (0·3–2·0 μmoles) did not stimulate succinate–neotetrazolium reductase. Formazan production was that expected from the sum of the individual contributions from succinate and reduced DPN (DPNH) (Fig. 2). This conclusion applied to fresh, aged (stored overnight at 0°) or frozen and thawed suspensions with various levels of added DPNH or DPN with β-hydroxybutyrate.

Effect of reducing agents. The results of adding cysteine, ascorbic acid or glutathione to the incubation medium are shown in Table 1. Although both cysteine and ascorbic acid lead to an increase in formazan production at low concentrations of tissue, the response remained non-linear.

Effect of metal ions. The addition of trace amounts of heavy-metal ions (copper, zinc) known to form mercaptides with sulphydryl groups produced large inhibition of the succinate–neotetrazolium reductase reaction. For instance, 5 μg. of Cu^{2+} ions caused almost complete inhibition of neotetrazolium reduction (Table 1). Similar amounts of Fe^{2+} ions were without effect (Table 1).

Evidence for cofactors. As the volume of the incubation mixture was decreased, the amount of formazan produced by a given amount of tissue increased (Fig. 3). This behaviour suggested that a cofactor was being diluted out on addition of the homogenate to the incubation mixture. The formazan produced by small amounts of fresh homogenate could be greatly increased by adding small volumes of boiled homogenate (1:10 sucrose homogenates boiled for 5 min., rehomogenized and made up to the original volume with water). Such boiled homogenates possessed very little succinate–neotetrazolium reductase activity (Fig. 4, curve *a*). Boiled homogenates, however, stimulated formazan production in the presence of fresh homogenate, indicating that the boiled homogenate contained a heat-stable cofactor for the reaction (Fig. 4, curves *b* and *c*). The stimulation by boiled homogenate was not marked at low levels of fresh homogenate (see curve *c*, Fig. 4), indicating that a heat-labile factor was also involved and which is supplied by fresh homogenate. The addition of ascorbic acid together with boiled homogenate to fresh homogenate, however, produced a linear response with increasing amounts of fresh homogenate (Fig. 4, curve *d*).

Effect of vitamin K_3. Menadione (vitamin K_3) produced very marked stimulation of the succinate–

neotetrazolium reductase reaction at low final concentrations (mM); the response remained non-linear with increasing amounts of fresh tissue added (Table 1). The stimulation by vitamin K_3 was virtually unaffected by the pre-incubation of homogenate and vitamin K_3 in the absence of neotetrazolium chloride. Evidence indicated a slight but variable increase in formazan production as the time of preincubation was increased up to 40 min. Formazan production by 10 mg. wet wt. of liver tissue was followed with varying preincubation periods and with mM-vitamin K_3 and an incubation time of 10 min. Formazan production after preincubation for 0, 20, 40 and 85 min. was 222, 240, 286, and 187 μg. of formazan, respectively.

The finding that preincubation with added vitamin K_3 did not greatly affect formazan production indicated that no permeability barrier was present between the added vitamin K_3 and the enzyme. This was further indicated by studying the effect of freezing and thawing and of high-speed blending on mitochondrial fractions. Disrupting the mitochondria by these methods did not increase the stimulation of the enzyme reaction by added vitamin K_3 (Table 1). Although vitamin K_3 had little influence on formazan production with small amounts of tissue, the addition of ascorbic acid produced a synergistic effect in that region. Ascorbic acid (1 μmole) and vitamin K_3 (1 μmole) in an incubation volume of 1 ml. led to a linear relationship between the extinction at 510 mμ and the amount of tissue added. For this effect, ascorbic acid was replaceable by cysteine in equivalent concentration. The addition of higher levels of ascorbic acid obscures the synergistic effect in homogenates as a result of the high blanks produced by both an enzymic and a non-enzymic interaction of neotetrazolium chloride and ascorbic acid; such blanks are very much smaller when the enzyme sample used is a mitochondrial suspension.

DISCUSSION

Since the succinate–neotetrazolium reductase enzyme sequence is associated with the mitochondrial fraction in rat-liver and rat-mammary-gland suspensions (Slater & Planterose, 1960) it is surprising that disruption of the mitochondria by a

Table 1. *Effects of various treatments and additives on formazan production by tissue suspensions*

Control samples were assayed as described in the Methods section except for results with EDTA and ox-plasma albumin; in these instances EDTA was omitted from the stock mixture used in the controls. All additions were made in 0·1 ml.; pre-incubation time was 2 min. Total volume of incubation mixture was 1·25 ml.

Tissue	Addition or treatment	Amount added	Incubation time (min.)	Formazan produced at three tissue concentrations (μg.) 5 mg.	10 mg.	15 mg.
Mouse liver	Tris buffer	—	10	4	23	64
	Untreated	—	10	4	17	51
	Freezing and thawing	—	10	12	26	55
	Untreated	—	10	15	26	78
Rat liver	EDTA	20 μmoles	10	15	39	67
	EDTA	150 μmoles	10	18	50	108
	Ox-plasma albumin	2 mg.	10	15	35	75
	Untreated	—	10	15	40	64
Rat liver	0·5 M-Sucrose	—	10	8	28	78
	0·25 M-Sucrose + 0·1 M-nicotinamide	—	10	7	29	54
	Untreated	—	10	7	27	59
Rat liver	Vitamin C	1 μmole	5	14	29	57
	Cysteine	1 μmole	5	10	21	43
	Glutathione	1 μmole	5	3	15	49
	Untreated	—	5	3	9	42
Rat liver	Cu^{2+} ions	0·5 μg.	10	7	23	48
	Cu^{2+} ions	5·0 μg.	10	4	7	12
	Fe^{2+} ions	5·0 μg.	10	8	28	40
	Untreated	—	10	11	26	45
Rat-liver mitochondria	Vitamin K_3	1 μmole	10	32	240	504
	Vitamin K_3 with freezing and thawing	1 μmole	10	19	209	360
	Untreated	—	10	10	23	37
Rat liver	Vitamin K_3	1 μmole	5	4	155	326
	Vitamin C	1 μmole	5	14	29	54
	Cysteine	1 μmole	5	10	21	44
	Vitamin K_3 + vitamin C	1 + 1 μmole	5	94	235	348
	Vitamin K_3 + cysteine	1 + 1 μmole	5	115	187	312
	Untreated	—	5	3	9	42

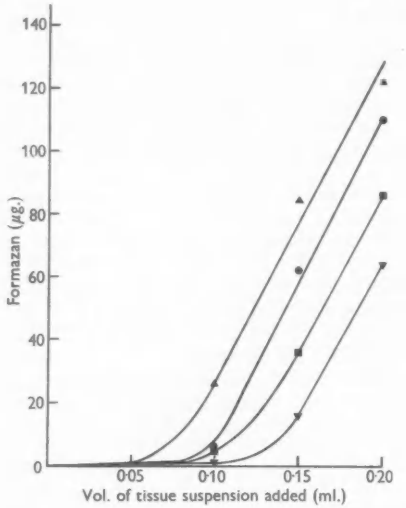

Fig. 3. Formazan production in the presence of varying volumes of stock mixture. Preincubation time was 2 min., incubation time 10 min.; 1·5 mg. of neotetrazolium chloride was present in each tube; rat-liver homogenate (1:10) was made in 0·25 M-sucrose. Curves ▲, ●, ■, ▼ were obtained with 1, 2, 3 and 4 ml. of stock solution, respectively.

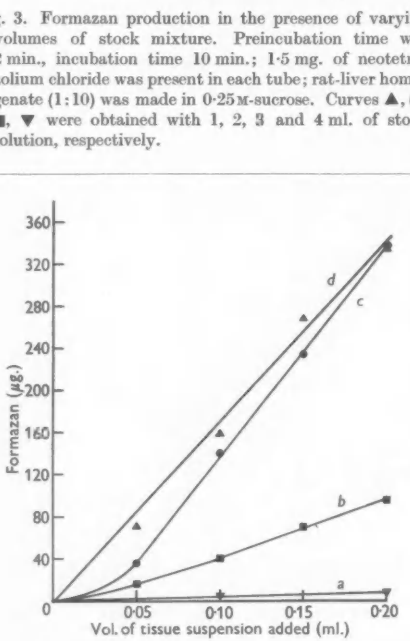

Fig. 4. Formazan production by rat-liver suspension (1:5) in 0·25 M-sucrose. Assay conditions were as described in the text; preincubation time was 2 min., incubation time 15 min. ▼, Activity of boiled homogenate; ■, activity of fresh homogenate; ●, activity of fresh homogenate + 67 mg. wet wt. of boiled tissue; ▲, as for ● but with 2 μmoles of vitamin C.

variety of treatments does not increase formazan production. This suggests that there is no permeability barrier to neotetrazolium chloride. Disruption of the mitochondrial fraction does not affect the manometric assay of succinic oxidase, or the reduction of neotetrazolium chloride with succinate as substrate (Shelton & Rice, 1957).

It was considered possible that the non-linear response might be overcome by adding large quantities of an enzymically inactive protein, i.e. ox-plasma albumin. This could fulfil three functions: (*a*) as a general remover of contaminants such as metal ions by adsorption; (*b*) as a stabilizer for small amounts of homogenate in relatively large volumes of stock solution; (*c*) as a source of trace quantities of certain cofactors (see e.g. Nason, Averbach & Terrell, 1956). As indicated by the results of this investigation, however, none of these factors appears responsible for the non-linear response. Similarly, the addition of EDTA was ineffective in improving the non-linear response. EDTA did, however, increase formazan production and this effect may be ascribed to the removal of metal ions (i.e. copper) which were shown to inhibit the reaction.

The non-linear response could be the result of contaminants in the experimental system used or the effect produced by diluting an essential cofactor. The latter alternative would appear to be the operative one for the following reasons. First, recrystallization of reagents, change of buffer, change in ionic strength of the incubating solution and addition of EDTA did not produce a linear response, which was, however, obtained with a Keilin–Hartree preparation under identical experimental conditions. Secondly, decreasing the volume of the incubation medium in the presence of a constant amount of tissue increased the amount of formazan produced. This increase was most marked when small amounts of tissue were used. This indicates that the final dilution of the tissue suspension is important, and contrasts with the behaviour of similar tissue suspensions used in the manometric assay of succinic oxidase where oxygen uptake increases linearly with increasing amounts of tissue. Thirdly, boiled homogenate, which was relatively inactive itself in the experimental system, greatly stimulated the activity of small quantities of fresh homogenate. This evidence suggests that the succinate–neotetrazolium reductase system is sensitive to dilution and contains a heat-stable cofactor essential for maximum activity.

The above-mentioned evidence for a cofactor in succinate–neotetrazolium reductase is in agreement with previous reports on the coupling of succinate oxidation with tetrazolium reduction. Bril (1954) showed that the activity of a homogenate decreased on prolonged dialysis and that after differential

centrifuging full activity could be obtained only by recombining the mitochondrial and soluble fractions. Bril's work therefore suggests that the enzyme system contains a water-soluble cofactor which, on homogenizing and centrifuging, is largely found in the soluble fraction. Sprinz & Waldschmidt-Leitz (1953) showed that boiled homogenate stimulated tetrazolium reduction by fresh tissue although they did not report on the activity of the boiled extract by itself. Sugimura & Ono (1956) have reported the presence of a cofactor in hot-water extracts of pigeon-breast muscle.

In contrast with these reports several investigations have indicated that succinate–neotetrazolium reductase was insensitive to dilution (Shelton & Rice, 1957; Kun & Abood, 1949). These workers reported a linear increase in formazan production with increasing amounts of tissue added; Kun & Abood (1949), however, obtained a very inefficient production of formazan. It was found possible to obtain a similar linear response by using a very dilute homogenate (0–0·5 ml. of 1:50 liver or mammary-gland homogenate) and a long incubation time; in these instances, also, formazan production was extremely inefficient. This suggests the possibility that neotetrazolium chloride can be reduced by two pathways, one of which is inefficient and insensitive to dilution, the other being much more efficient in terms of μg. of formazan produced/mg. of tissue, but is correspondingly affected by dilution. This paper is concerned solely with the second possible pathway of reduction.

Various attempts to elucidate the general nature of the heat-stable cofactor are reported in the Results section. An obvious possibility was flavine mononucleotide (FMN) since several reports have shown that tetrazolium reduction occurs in many systems via a flavoprotein enzyme (Brodie & Gots, 1951; Kun, 1951; Shelton & Schneider, 1952). However, FMN with or without preincubation with ATP and homogenate was without effect on the non-linear response. Similarly, low concentrations of iron and copper which are often found in essential association with flavoenzymes (Nicholas, 1957) did not induce a linear response, but copper, on the contrary, caused intense inhibition. Metal-ion inhibition is often the result of combination between the metal ion and essential sulphydryl groups. Both ascorbic acid and cysteine, which are effective in protecting sulphydryl groups against metal-ion inhibition and oxidation, caused considerable increase in formazan production; glutathione, very surprisingly, did not produce this effect. The combination of reducing agent and boiled homogenate produced a linear response with increasing amounts of fresh homogenate. This suggests the possibility that the non-linear response is the result of a dilution of a heat-stable cofactor found in boiled homogenate and a heat-labile cofactor which protects the sulphydryl groups of the enzyme system. The heat-labile factor can apparently be replaced by ascorbic acid or cysteine.

Vitamin K_3 stimulates the succinate–neotetrazolium reductase system above a certain limiting concentration of tissue below which it has little effect. This again suggests the possibility that two factors are operative in producing the non-linear response. Since the stimulation produced by vitamin K_3 is remarkably similar to that produced by boiled homogenate, it is possible that vitamin K_3 is acting in a similar fashion to the heat-stable factor mentioned previously. It is also possible that vitamin K_3 is creating an entirely artificial electron pathway of its own. Quinones have become increasingly associated with pathways of electron transfer. In particular, several reports have indicated a role for a quinone in the respiratory chain (Colpa-Boonstra & Slater, 1958; Martius, 1954). Investigations on the pharmacological activity of several quinones (Herz, 1954) have shown that some possess a stimulatory effect on tetrazolium reduction. Vitamin K_3 is heat-stable, and although it is concentrated in the particulate fractions of the cell (Martius, 1956) it does not appear to be strongly bound to the mitochondria as does vitamin K_1 (Martius & Nitz-Litzow, 1954; Schulz & Goss, 1956). As the effects produced by boiled homogenate and vitamin K_3 are similar, and as these effects are similarly affected by cysteine or ascorbic acid, it seems possible that the heat-stable cofactor present in boiled homogenates is a quinone replaceable by vitamin K_3. From the indications that a quinone group and sulphydryl groups are essential for an efficient production of formazan, it is worth noting that 2-substituted naphthaquinones like vitamin K_3 react readily with sulphydryl compounds to give 2:3-disubstituted naphthaquinones (Thomson, 1957). The natural 2:3-disubstituted naphthaquinone vitamin K_1, which does not react with sulphydryl compounds, is inactive in stimulating succinate–neotetrazolium reductase (unpublished results).

The influence of added DPN is of special interest in view of several reports about its possible participation in succinate–neotetrazolium reductase. Sprinz & Waldschmidt-Leitz (1953) showed that liver homogenates could catalyse formazan production in the presence of relatively large amounts of DPN (approximately 3 mM); similarly, both Barker (1953) and Zöllner & Rothemund (1954) showed that formazan production in homogenates was stimulated by added DPN. Bril (1954) reported that succinate–neotetrazolium reductase decreased on dialysis but that formazan production was restored at least partly by added DPN. Sourkes & Lagnado (1957), using washed mitochondria or

solubilized enzyme systems from rat liver, have shown that certain purines including DPN act as cofactors in succinate–neotetrazolium reductase; small amounts of added DPN (0·1–0·3 μmole) stimulated formazan production in the presence of succinate some five-fold. Zöllner & Rothemund (1954), however, reported no synergistic stimulation of formazan production when DPN was incubated in the presence of succinate anaerobically.

In the experiments reported in this paper there was no synergistic stimulation of succinate–neotetrazolium reductase by added DPN. DPN in the presence of suitable substrate (endogenous or added β-hydroxybutyrate) produces DPNH which then led to formazan production. DPNH does not reduce neotetrazolium chloride directly (Slater, 1959*a*). The joint addition of DPNH and succinate did not lead to any significant extra formazan formation over that expected from the sum of the individual contributions alone (Fig. 2). This conclusion applied to fresh homogenate and fresh mitochondrial suspensions, to homogenates and mitochondrial suspensions which had been frozen and thawed, and to homogenates and mitochondrial suspensions which had been aged at 0° or at 37° in the absence of added substrate, i.e. succinate or DPN under aerobic conditions.

It appears therefore that DPNH does not stimulate succinate–neotetrazolium reductase under aerobic conditions. It seems likely that the formazan produced by DPNH is formed by DPNH oxidation via an enzyme of a type similar to, if not identical with, the quinone reductase described by Martius & Strufe (1954); this type of enzyme is known to be associated with the particulate material of the cell (Slater, 1959*b*).

SUMMARY

1. The enzyme system linking succinate oxidation with neotetrazolium reduction has been investigated in rat-liver homogenates. In unsupplemented homogenates the production of formazan did not increase proportionately with increasing amounts of tissue. This non-linear response with succinate as substrate was unaffected by: freezing and thawing the suspension; high-speed blending; homogenizing in hyper-osmotic sucrose; homogenizing in the presence of nicotinamide; addition of EDTA, ox-plasma albumin, lipoic acid or riboflavin 5′-phosphate to the incubation medium.

2. The variations produced in formazan production (*a*) on varying the volume of the incubation medium and (*b*) on adding boiled homogenate to the incubation medium suggested that a water-soluble cofactor was involved.

3. Formazan production was stimulated by the addition of vitamin K_3 to the incubation medium; this stimulation was, however, small with low final concentrations of tissue.

4. Ascorbic acid or cysteine synergistically increased formazan production in the presence of vitamin K_3 especially with low final concentrations of tissue.

5. It is suggested that for optimum formazan production a heat-stable cofactor and reduced sulphydryl groups are required. The heat-stable cofactor appears to resemble vitamin K_3 but not vitamin K_1 in its properties; ascorbic acid and cysteine appear to function in the system by maintaining sylphydryl groups in the reduced form.

6. Diphosphopyridine nucleotide does not appear to be directly involved in the succinate–neotetrazolium reductase system.

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Chromatographic Studies With Pig Adrenocorticotrophin

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Oxycellulose concentrates of adrenocorticotrophin have been chromatographed on carboxylic-cation exchangers by a number of workers. Buffered Amberlite IRC-50 was used by White & Fierce (1953), Li, Geschwind, Dixon, Levy & Harris (1955), Dixon & Stack-Dunne (1955) and Dedman, Farmer & Morris (1957). Raben (1956) published a note on the use of carboxymethylcellulose and M-acetic acid in conjunction with a calcium chloride gradient. With concentrates obtained from pig pituitary glands, Dixon & Stack-Dunne (1955) obtained evidence for the presence of three active components which were designated corticotrophin A_1, A_2 and A_3, and further showed that alkali probably converted corticotrophin A_1 into A_2 by the hydrolysis of an amide group. Shepherd *et al.* (1956), from countercurrent-distribution studies, reported the presence of seven corticotrophins in oxycellulose concentrates. Partition chromatography utilizing an ammonium sulphate–zinc sulphate-2-ethoxyethanol–water system has given two highly active peaks (T. H. Farmer & C. J. O. R. Morris, unpublished work).

This paper reports the use of carboxymethylcellulose and diethylaminoethylcellulose in the chromatography of oxycellulose concentrates of pig adrenocorticotrophin.

EXPERIMENTAL

Preparation of crude corticotrophin. The oxycellulose (OC) concentrates were prepared, according to the method of Astwood, Raben, Payne & Grady (1951), from acetone-dried powder made from the anterior lobes of pig pituitary glands (Dedman *et al.* 1957).

Carboxymethyl- and diethylaminoethyl-cellulose. These ion-exchange reagents were prepared from 200–400 mesh, dry-sieved, Whatman ashless paper powder, standard grade, as described by Peterson & Sober (1956).

Buffer solutions. Sodium acetate, pH 5·9: sodium acetate ($CH_3 \cdot CO_2Na, 3H_2O$; 28·6 g.) with 5·45 ml. of 2 N-acetic acid was made up to 2 l. with water. Sodium phosphate, pH 5·5: 950 ml. of 0·1 N-NaH_2PO_4 was mixed with 100 ml. of 0·05 M-Na_2HPO_4. Sodium phosphate, pH 6·3: 80 ml. of 0·1 M-NaH_2PO_4 and 20 ml. of 0·1 M-Na_2HPO_4 were diluted to 1 l. with water. Sodium phosphate, pH 11·3: 50 ml. of 0·05 M-Na_2HPO_4 was added to 7·6 ml. of 0·1 N-NaOH. α-Picoline acetate, pH 5·7: 61 g. of α-picoline, distilled from BaO, together with 231 ml. of 2 N-acetic acid was diluted to 2 l. with water.

Ion-exchange chromatography. Buffered carboxymethyl (CM)-cellulose was obtained by first converting the ion exchanger into its sodium salt by the addition of 7 ml. of 0·1 N-NaOH/g. of CM-cellulose, and then suspending this material several times, in portions of the relevant buffer solution. With the α-picoline acetate system the CM-cellulose was washed initially with aqueous 0·3 % α-picoline before suspension in buffer solution. Diethylaminoethyl (DEAE)-cellulose was shaken with 0·1 M-phosphate buffer, pH 6·3, before being suspended in 0·01 M-phosphate, as equilibration with the dilute buffer solution took a considerable time. It was found necessary with both CM-cellulose and DEAE-cellulose to use a glass homogenizer to break up small aggregates of the exchanger. Columns were packed under a pressure of 15 cm. Hg, either in sections or as suggested by Porath (1956); there was no observed difference in the performance of columns packed by these two procedures. Before use columns were washed with buffer solution until the pH of the effluent was within 0·02 unit of pH of the developing solution. This usually took 24–72 hr., depending on the size of the column. To minimize the growth of micro-organisms in phosphate buffer solutions the solutions were saturated with toluene, and fractions were collected into tubes containing sufficient HCl to adjust to pH 3·0. The sample to be chromatographed was dissolved in the minimum volume of buffer solution by shaking for 20 min., any insoluble material being removed by centrifuging. Columns were developed under a constant head of buffer solution, flow rates of less than 25 ml./cm.²/hr. giving the best results. $E_{1\,cm.}$ at 275 mμ was used to estimate the amounts of various fractions eluted from columns with the sodium acetate and sodium phosphate buffer solutions. The α-picoline acetate buffer solution absorbed strongly at 275 mμ and $E_{1\,cm.}$ at 285 mμ had to be used. All extinctions were measured relative to the buffer solutions with a Unicam SP. 500 spectrophotometer.

Oxidation of corticotrophin A_1. Corticotrophin A_1 was oxidized by aqueous H_2O_2 as described by Dedman *et al.* (1957).

Reduction of oxidized corticotrophin A_1. Oxidized corticotrophin A_1 was reduced with cysteine as described by Dedman *et al.* (1957).

Action of sodium phosphate solution, pH 11·3, *on fraction* 4 *and fraction* 5 (A_1) *from column* 4. Solutions of 2·7 mg. of fraction 4 and 3·6 mg. of fraction 5 in 0·8 ml. of sodium phosphate solution, pH 11·3, were allowed to stand under N_2 at 4° for 15 hr. and were then adjusted to pH 5·5 by the addition of M-H_3PO_4.

Bioassay. The adrenocorticotrophic potency was measured by the adrenal ascorbic acid-depletion method of Sayers, Sayers & Woodbury (1948), as modified by Morris (1951). The results, summarized in Table 1 show the mean depletions of four rats.

Desalting. Salts were removed from solutions by the use of Dowex-50(×16) and Dowex-2(×8) as described by Dedman *et al.* (1957).

RESULTS

Fig. 1 (column 1) illustrates the resolution obtained by chromatographing an OC concentrate on a preparative column in sodium acetate buffer, pH 5·9. The faster-running components were better separated than on Amberlite IRC-50, whereas the two major peaks, fractions 4 and 5, showed a resemblance to those obtained with Amberlite IRC-50 chromatography. The material corresponding to these peaks was highly potent in the adrenal ascorbic acid-depletion assay (Table 1). Fractions 2 and 3 were desalted, freeze-dried, and rechromatographed on CM-cellulose in sodium phosphate buffer, pH 5·5; the results are shown in Fig. 1 (columns 2 and 3, respectively). Fractions 1–4 from column 2 were weakly active biologically, fractions 5 and 6 being moderately active. Fractions 1, 2 and 3 from column 3 were highly potent (Table 1).

To eliminate possible hydrolytic effects of unbuffered Dowex-50(×16) in the desalting procedure [Amberlite IR 120 having been reported to hydrolyse insulin (Davies & Harris, 1958)], it was thought desirable to use a volatile buffer solution. Dixon (1956) had successfully used pyridine acetate in conjunction with Amberlite IRC-50, and α-picoline acetate, pH 5·7, was found suitable for use with CM-cellulose. Fig. 2 shows the results obtained with a preparative column. The picture was similar to that obtained with column 1 although the resolution appeared to be of a lower order. The effluent was bulked as indicated, freeze-dried and the fractions were assayed for biological activity. Fractions 4 and 5 were highly active; fraction 3 was moderately active, whereas fractions 1 and 2 had negligible activity (Table 1). Samples of these fractions were then rechromatographed on CM-cellulose in the sodium phosphate buffer, pH 5·5. The results are summarized in Fig. 3. The first two peaks in

Table 1. *Adrenal ascorbic acid depletions produced by chromatographic fractions*

Column no.	Fraction	Dose (μmg./100 g.)	Mean ascorbic acid depletion (μg./100 mg. of adrenal tissue) (±S.E.)
1	4	10	36 (±3)
	5	10	61 (±10)
2	1	25	Inactive
	2	25	17 (±4)
	3	25	19 (±7)
	4	25	40 (±8)
	5	25	50 (±6)
	6	25	94 (±10)
3	1	25	125 (±5)
	2	25	100 (±12)
	3	25	87 (±9)
4	1	25	Inactive
	2	25	Inactive
	3	10	25 (±6)
	4	10	93 (±10)
	5	10	106 (±13)
10	A_1	10	48 (±13)
12	A_2	10	49 (±6)
	A_1	10	74 (±8)
13	A_2	20	87 (±9)
	A_{1a}	20	95 (±12)
14	A_3	18	80 (±17)
	A_2	24	125 (±14)
21	1	20	Inactive
	1 (reduced)	10	85 (±11)
	2	20	120 (±5)
	2 (reduced)	10	92 (±12)

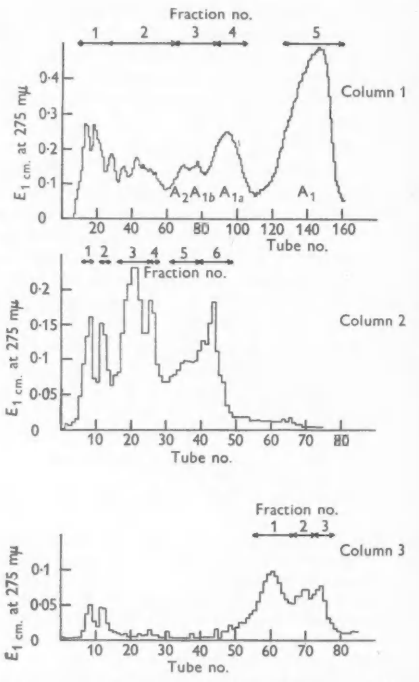

Fig. 1. Column 1: 5 g. of CM-cellulose, 42·5 cm. × 1·2 cm., sodium acetate, pH 5·9; 100 mg. of OC concentrate in 10 ml. of buffer solution was used and 3·6 ml. fractions were collected. Column 2: 1·5 g. of CM-cellulose, 90·6 cm. × 0·35 cm., sodium phosphate, pH 5·5; 5·9 mg. of fraction 2 from column 1 in 1 ml. of buffer solution was used and 1·55 ml. fractions were collected. Column 3: 1·5 g. of CM-cellulose, 90·6 cm. × 0·35 cm., sodium phosphate, pH 5·5; 4·8 mg. of fraction 3 from column 1 in 1 ml. of buffer solution was used and 1·55 ml. fractions were collected.

columns 6–9 were due to a non-retarded substance, possibly an α-picoline oxidation product, and α-picoline, respectively. The surprising heterogeneity of fraction 4 from column 4 is shown in column 8.

In an attempt to clarify the relationships between fractions 5, 4 and 3 from column 4 and corticotrophins A_1, A_2 and A_3, fractions 5 and 4 from column 4 were treated with sodium phosphate buffer (Dixon & Stack-Dunne, 1955), pH 11·3, and rechromatographed on CM-cellulose in sodium phosphate buffer, pH 5·5. Figs. 4 and 5 summarize the results. The alkaline buffer solution appears to have transformed approximately 50 % of fraction 5 into a component with a retention volume equal to that of the faster-running component of fraction 4. Fig. 5 illustrates the effect of the pH 11·3 buffer solution on fraction 4 from column 4; fraction 3 from column 4 was chromatographed for comparison. Here it was seen that the more retarded component of fraction 4 was converted into material which was chromatographically similar to the less-retarded component, together with another faster-running substance. Both products had high biological activity (Table 1). Column 15 shows the results obtained on chromatographing a sample of the moderately active fraction 3 from column 4.

For comparison, fractions designated A_1, A_2 and A_4, which had been obtained by chromatographing OC concentrates on Amberlite IRC-50 at pH 8·3 (Dedman *et al.* 1957), were analysed on CM-cellulose columns with sodium phosphate buffer, pH 5·5. Columns 16 and 17 revealed the heterogeneous nature of the A_4 and A_2 fractions, respectively, whereas column 18 showed that the A_1 fraction contained only one major component (Fig. 6). These results are summarized in Table 2.

The possibility of using DEAE-cellulose as an alternative method for fractionating corticotrophin concentrates was investigated, 0·01 M-sodium phosphate, pH 6·3, being used. Fig. 7 (column 19) shows some typical results. Biological activity was distributed throughout the chromatogram, the last two peaks exhibiting moderate activity. However, large losses in biological activity were sustained and this method was discarded for preparative purposes. A sample of fraction 5 from column 1 was analysed in this system and gave the symmetrical peak shown in Fig. 8. The resolving power of the DEAE-cellulose

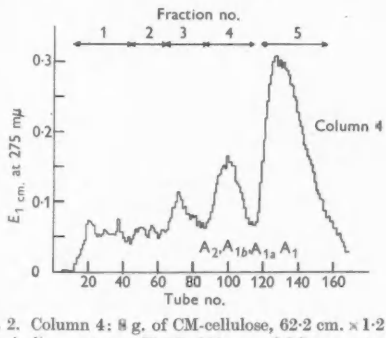

Fig. 2. Column 4: 8 g. of CM-cellulose, 62·2 cm. × 1·2 cm., α-picoline acetate, pH 5·7; 100 mg. of OC concentrate in 12 ml. of buffer solution was used and 3·6 ml. fractions were collected.

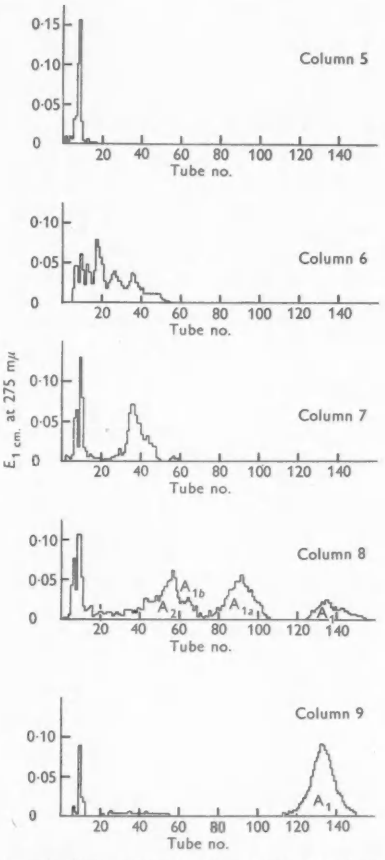

Fig. 3. CM-cellulose (1·35 g.) column, 79·2 cm. × 0·35 cm., sodium phosphate, pH 5·5; 1·55 ml. fractions were collected. Column 5: 1·3 mg. of fraction 1 from column 4 in 0·5 ml. of buffer solution. Column 6: 1·5 mg. of fraction 2 from column 4 in 0·8 ml. of buffer solution. Column 7: 3·0 mg. of fraction 3 from column 4 in 1·0 ml. of buffer solution. Column 8: 4·1 mg. of fraction 4 from column 4 in 2·0 ml. of buffer solution. Column 9: 2·2 mg. of fraction 5 from column 4 in 1·0 ml. of buffer solution.

columns, as calculated by the method of Simpson & Wheaton (1954), was approximately 4 plates/cm. compared with 20 plates/cm. obtained with the CM-cellulose columns. It was, however, decided that the behaviour of a mixture of oxidized and non-oxidized corticotrophin A_1 on a DEAE-cellulose column might prove instructive. These substances had been previously separated on Amberlite IRC-50 (Dixon & Stack-Dunne, 1955) and CM-cellulose (Farmer & Morris, 1956). Fig. 9 illustrates the asymmetrical peak obtained. The effluent was bulked as indicated and assayed before and after reduction with cysteine. Fraction 1 was inactive before, but was highly active after cysteine treatment, whereas fraction 2 was highly active before reduction (Table 1).

DISCUSSION

In agreement with the results of previous investigators (White & Fierce, 1953; Li *et al.* 1955, Dixon & Stack-Dunne, 1955; Dedman *et al.* 1957) chromatography of OC concentrates of pig adrenocorticotrophin on CM-cellulose has shown that the major active component accounted for approximately 30% of the total material. In addition, evidence has been obtained for the presence of three other highly active components (Fig. 1, columns 1 and 3). The major component corresponded to corticotrophin A_1, as was shown by column 18. Fraction 4 from column 4 was found to be complex, three peaks other than one due to A_1 were obtained on chromatography in the sodium phosphate buffer, pH 5·5 (column 8). Columns 12 and 14 indicated that the fastest-running peak corresponded to A_2, and the other two were therefore named A_{1a} and A_{1b}, respectively. As it has been suggested by Dixon & Stack-Dunne (1955) that the conversion of A_1 into A_2 was accompanied by the hydrolysis of an amide group, and since A_{1a} and A_{1b} were also converted into less-retarded substances by the action of alkali (columns 12 and 14), it seemed probable that A_{1a} and A_{1b} were also amides. Bell, Howard, Shepherd, Finn & Meisenhelder (1956) have shown that biologically active molecules containing 28, 30 and 31 amino acid residues may be prepared from the 39 amino acid residue β-corticotrophin by pepsin digestion, the *C*-terminal sequence being attacked under these conditions. The amino acid composition of corticotrophin A_1, obtained from Amberlite IRC-50

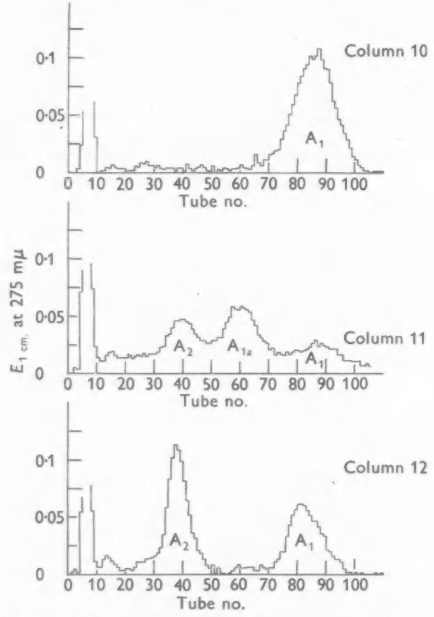

Fig. 4. CM-cellulose (0·9 g.) column, 40·0 cm. × 0·5 cm., sodium phosphate, pH 5·5; 1·55 ml. fractions were collected. Column 10: 3·0 mg. of fraction 5 from column 4 in 0·6 ml. of buffer solution. Column 11: 3·0 mg. of fraction 4 from column 4 in 0·6 ml. of buffer solution. Column 12: 3·6 mg. of fraction 5 from column 4, pH 11·3, for 15 hr.

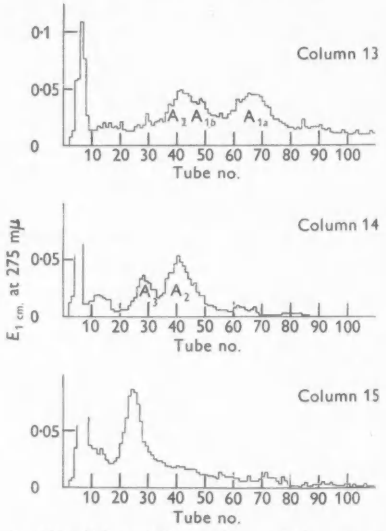

Fig. 5. CM-cellulose (0·9 g.) column, 40·0 cm. × 0·5 cm., sodium phosphate, pH 5·5; 1·55 ml. fractions were collected. Column 13: 2·7 mg. of fraction 4 from column 4 in 0·6 ml. of buffer solution. Column 14: 2·7 mg. of fraction 4 from column 4, pH 11·3, for 15 hr. Column 15: 2·8 mg. of fraction 3 from column 4 in 0·6 ml. of buffer solution.

Table 2. *Retention of corticotrophin peaks (columns 10–15, 17, 18)*

The effluent volumes refer to a CM-cellulose column, 40·0 cm. × 0·5 cm., with sodium phosphate, pH 5·5, as eluent.

Material chromatographed	Effluent vol. to peak maxima (ml.)				
Fraction 5 from column 4	—	—	—	—	149
Fraction 5 from column 4, pH 11·3	—	—	59	—	143
Fraction 4 from column 4	—	—	65	104	—
Fraction 4 from column 4, pH 11·3	—	45	64	—	—
Fraction 3 from column 4	39	—	—	—	—
Corticotrophin A_2*	37	—	67	101	—
Corticotrophin A_1*	—	—	—	—	138

* Obtained by chromatography of OC concentrates on Amberlite IRC-50 (Dedman, Farmer & Morris, 1957).

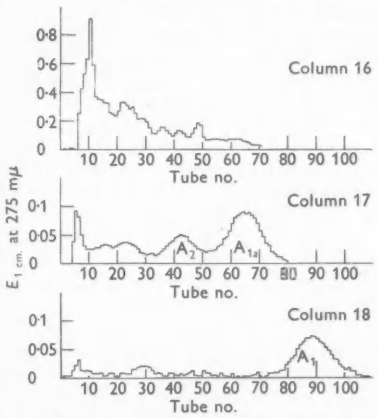

Fig. 6. Column 16: 4·5 g. of CM-cellulose, 86·5 cm. × 0·7 cm., sodium phosphate, pH 5·5; 28·5 mg. of A_4 (from a column of Amberlite IRC-50) was chromatographed in 3 ml. of buffer solution and 3·6 ml. fractions were collected. Columns 17 and 18: 0·9 g. of CM-cellulose, 40·0 cm. × 0·5 cm., sodium phosphate, pH 5·5; 1·55 ml. fractions were collected. Column 17: 6·2 mg. of A_2 (from a column of Amberlite IRC-50) in 1·3 ml. of buffer solution. Column 18: 1·7 mg. of A_1 (from a column of Amberlite IRC-50) in 0·5 ml. of buffer solution.

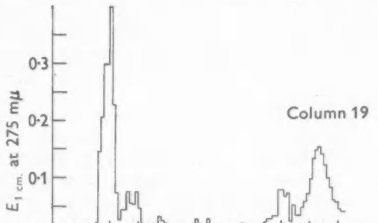

Fig. 7. Column 19: 7·5 g. of DEAE-cellulose, 61·2 cm. × 0·9 cm., 0·01 M-sodium phosphate, pH 6·3; 10 mg. of OC concentrate was chromatographed in 1 ml. of buffer solution and 1·55 ml. fractions were collected.

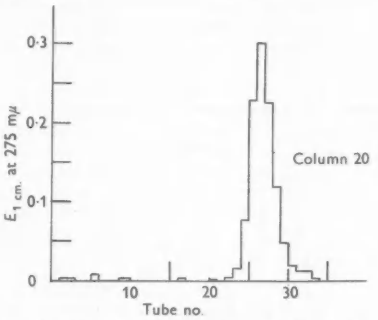

Fig. 8. Column 20: 2 g. of DEAE-cellulose, 62·8 cm. × 0·5 cm., 0·01 M-sodium phosphate, pH 6·3; 1·9 mg. of A_1 was chromatographed in 0·5 ml. of buffer solution and 1·55 ml. fractions were collected.

chromatography differed only slightly from that of β-corticotrophin (Dedman *et al.* 1957), and it seemed likely that corticotrophins A_{1a} and A_{1b} differed from A_1 in the *C*-terminal sequences. Further studies are needed to elucidate this point.

The action of alkali on fraction 4 from column 4 produced another highly active corticotrophin which was probably identical with corticotrophin A_3. This material had a retention volume similar to that of fraction 3 from column 4 (Fig. 5), and it appeared probable that the activity of fraction 3 was due to the presence of A_3, since A_3 had been detected on OC concentrates by Dixon & Stack-Dunne (1955). The action of alkali on A_1 did not produce material similar to A_3 (column 12), and this result suggested that A_3 was a product of A_{1a} or A_{1b}. However, no evidence has been obtained to support this suggestion.

Chromatography of fractions designated corticotrophins A_1 and A_2, and fraction A_4, obtained from columns of Amberlite IRC-50 (Dedman *et al.* 1957), gave the results illustrated in Fig. 6. A_1 was found to contain one major component chromatographically identical with the A_1 obtained from CM-cellulose chromatography. However, the A_2 fraction obtained from Amberlite IRC-50 columns gave a picture similar to that obtained with fraction 4

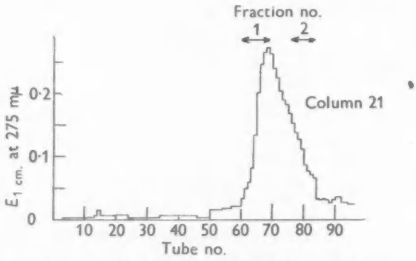

Fig. 9. Column 21: 6 g. of DEAE-cellulose, 85·6 cm. × 0·7 cm., in 0·01 M-sodium phosphate, pH 6·3; the mixture of 4 mg. of oxidized A_1 and 3·1 mg. of A_1 was chromatographed in 0·7 ml. of buffer solution and 1·55 ml. fractions were collected.

from column 4, indicating that corticotrophin A_{1a} and A_2 were present. The term corticotrophin A_2 should therefore be used for material which has the same retention volume, when chromatographed on CM-cellulose in the sodium phosphate buffer, pH 5·5, as the product formed on the treatment of corticotrophin A_1 with alkali. As was expected, fraction A_4, the least-retarded material on Amberlite IRC-50 at pH 8·3, proved to be composed of many components.

A recent publication by Farrell *et al.* (1958) has reported that Bell's δ_1-corticotrophin (Shepherd *et al.* 1956) was more active than β-corticotrophin in mobilizing aldosterone in the decerebrate dog. In the light of these results, it would be interesting to know which fraction obtained from the CM-cellulose chromatography of OC concentrates corresponded to δ_1-corticotrophin.

DEAE-cellulose columns, run in 0·01 M-sodium phosphate buffer, pH 6·3, resolved OC concentrates into a number of peaks. However, although activity was distributed throughout the chromatogram, a large loss of biological activity made this system unsuitable for preparative purposes. The most active peak had the greatest retention volume. Chromatography of a sample of fraction 5 from column 1 (A_1) in this system gave a single peak, as illustrated in Fig. 8. An attempt to separate oxidized and non-oxidized A_1 was partially successful, although two peaks were not obtained. In this experiment the oxidized A_1 ran faster than the non-oxidized A_1, as on CM-cellulose, indicating that mechanisms other than ion exchange were contributing to the process of chromatography.

SUMMARY

1. Carboxymethylcellulose has been used to prepare corticotrophin A_1 and show that at least three other chromatographically distinct corticotrophins are present in oxycellulose concentrates. One of these has been identified with corticotrophin A_2 and the other two have been named corticotrophins A_{1a} and A_{1b}.

2. In agreement with previous workers alkali has been shown to convert corticotrophin A_1 into A_2, and corticotrophins A_{1a} and A_{1b} into less-retarded substances on carboxymethylcellulose columns. One of these products has been tentatively identified with corticotrophin A_3.

3. Diethylaminoethylcellulose has been used to fractionate oxycellulose concentrates, although large losses in biological activity were observed. Corticotrophin A_1 behaved as a homogeneous substance on diethylaminoethylcellulose, and oxidized A_1 and A_1 were partially separated.

The author wishes to thank Professor C. J. O. R. Morris for helpful discussions, and Miss Margaret Dedman and Miss Gladys Atfield for performing bioassays on samples.

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Mammalian Glycosidases

2. PROPERTIES OF α-MANNOSIDASE AND β-GALACTOSIDASE FROM RAT EPIDIDYMIS*

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In a study of the occurrence of various glycosidases in mammalian tissues, the enzymes α-mannosidase and β-galactosidase were found to be widely distributed throughout the tissues of the mouse and the rat, particularly rich sources being the epididymis of these animals (Conchie, Findlay & Levvy, 1959*a*). Investigations have since been extended to the epididymis of other animals (Conchie, Findlay & Levvy, 1959*b*), but rat epididymis remains the best source of these two enzymes.

The present paper is concerned with some properties of α-mannosidase and β-galactosidase from this source, the chromogenic glycosides *p*-nitrophenyl α-mannoside, phenyl α-mannoside, *o*-nitrophenyl β-galactoside and phenyl β-galactoside being used as substrates. Some observations on mammalian β-galactosidase, in particular the enzyme from rat kidney, have been made by Cohen, Tsou, Rutenburg & Seligman (1952), using the chromogenic substrate 6-bromo-2-naphthyl β-galactoside.

EXPERIMENTAL

Materials

p-Nitrophenyl α-D-mannoside and D-mannono-1 → 4-lactone were prepared as described by Conchie *et al.* (1959*a*).

Phenyl α-D-mannoside, prepared as described by Helferich & Winkler (1933), had m.p. 131–132° and $[\alpha]_D^{17}$ +113° in water (*c*, 1·0).

o-Nitrophenyl β-D-galactoside was prepared by the method of Seidman & Link (1950), and had m.p. 193° and $[\alpha]_D^{16}$ − 51° in water (*c*, 1·0).

Phenyl β-D-galactoside was prepared as described by Helferich (1944), and had m.p. 155–156° and $[\alpha]_D^{16}$ − 42° in water (*c*, 1·0).

Galactono-1 → 4-lactone was obtained from L. Light and Co. Ltd.

Enzyme preparations

Adult rat epididymis was the source of all the enzyme preparations used in the experiments described below. Complete epididymides were homogenized in water (0·6 g. in 10 ml.) with a glass homogenizer (Jencons Ltd.), and incubated for 1 hr. at 37° in 0·1 M-acetic acid–NaOH buffer, pH 5·0. The suspension was centrifuged at 1500 *g* for 15 min. and the resulting supernatant fractionated with $(NH_4)_2SO_4$, pH 5·0, the precipitate obtained between 20 and 80% saturation limits being collected at 10 000 *g* and dissolved in water (10 ml.). Such a stock solution could be kept for only a few days at 0° without an appreciable loss in α-mannosidase activity. On the other hand, even after several weeks at 0° there was little loss in β-galactosidase activity. For normal assay purposes the solution had to be diluted before use to liberate suitable amounts of aglycone (60–120 μg. of *p*-nitrophenol, 80–140 μg. of *o*-nitrophenol and 30–50 μg. of phenol). Typical dilutions for α-mannosidase were 1:25 and 1:10, and for β-galactosidase 1:10 and 1:5, for the nitrophenyl and phenyl substrates respectively.

Enzyme assays

Liberation of phenol. Liberation of phenol from phenyl α-mannoside and phenyl β-galactoside was estimated as by the procedure of Kerr, Graham & Levvy (1948), the colour intensity being measured on the Spekker photoelectric absorptiometer with Ilford 608 red filters (peak transmission 680 mμ). Incubation of enzyme with substrate was for 1 hr. at 37° in 0·1 M-acetic acid–NaOH buffer, pH 5·0 for α-mannosidase assays and pH 2·9 for β-galactosidase assays. The incubation mixture contained 0·5 ml. of enzyme preparation in a final volume of 1 ml. Except where otherwise stated, the substrate concentration was 10 mM for phenyl α-mannoside and 5 mM for phenyl β-galactoside.

Liberation of p-nitrophenol. Liberation of *p*-nitrophenol from *p*-nitrophenyl α-mannoside was measured as described by Conchie *et al.* (1959*a*). Except where otherwise stated, incubation of enzyme with substrate was for 1 hr. at 37° in 0·125 M-acetic acid–NaOH buffer, pH 5·0, the substrate concentration being 6 mM. The incubation mixture contained 0·5 ml. of enzyme preparation in a final volume of 4 ml.

Liberation of o-nitrophenol. Liberation of *o*-nitrophenol from *o*-nitrophenyl β-galactoside was measured as described by Conchie *et al.* (1959*a*). Except where otherwise stated, incubations were for 1 hr. at 37° in 0·125 M-acetic acid–NaOH buffer, pH 2·9, the substrate concentration being 2·5 mM.

RESULTS

Effect of pH

α-Mannosidase. The variation in relative activity with pH of an enzyme preparation [20–80% $(NH_4)_2SO_4$ fraction] over the range pH 2·5–7·5 is shown for the hydrolysis of 6 mM-*p*-nitrophenyl

* Part 1: Conchie, Findlay & Levvy (1959*a*).

α-mannoside in 0·125M-acetic acid–NaOH buffer (Fig. 1), and 10 mM-phenyl α-mannoside in 0·1M-acetic acid–NaOH buffer (Fig. 2). In each a smooth curve with a sharp peak at pH 5·0 resulted. Further determinations, with 6 mM-*p*-nitrophenyl α-mannoside as substrate, were made in 0·125M-citric acid–NaOH buffer and phosphate–citrate buffer (McIlvaine, 1921), as well as in 0·125M-acetic acid–NaOH buffer with substrate concentrations of 2 and 12 mM. In all cases, curves similar to that in Fig. 1 were obtained. Measurements in all the above-mentioned buffers with unfractionated buffer extracts of crude homogenates also gave similar curves. Enzyme preparations assayed at pH 5·0 in 0·125M-acetic acid–NaOH, 0·125M-citric acid–NaOH and phosphate–citrate buffers showed very little variation in their total activity with the nature of the buffer.

β-Galactosidase. Figs. 1 and 2 also show the pH-activity curves of the same enzyme preparation for the hydrolysis of 2·5 mM-*o*-nitrophenyl β-galactoside and 5 mM-phenyl β-galactoside respectively. In acetic acid–NaOH buffer, the pH optimum for the hydrolysis of the nitrophenyl substrate was at 2·9, whereas for the phenyl substrate two optima, at 2·9 and 3·5, were obtained. In phosphate–citrate and in citric acid–NaOH buffers, however, the same two optima occurred with the nitrophenyl substrate. Results identical with the above were obtained when the supernatant from a buffer extract of a crude homogenate was used as the enzyme source. Determinations with 7·5 mM-*o*-nitrophenyl β-galactoside in 0·125M-acetic acid–NaOH buffer gave a curve similar to that obtained with a substrate concentration of 2·5 mM. Comparisons of enzyme activity in 0·125M-acetic acid–NaOH buffer, pH 2·9, 0·125M-citric acid–NaOH buffer, pH 3·6, and McIlvaine's phosphate–citrate buffer, pH 3·6, with the *o*-nitrophenyl substrate, showed the total activities to be within 8 % of each other. Acetic acid–NaOH buffers, pH 2·9, were employed for all the remaining experiments with β-galactosidase described in this paper.

Cohen *et al.* (1952), in their study on rat-kidney β-galactosidase with 6-bromo-2-naphthyl β-galactoside in phosphate–citrate buffer, found the optimum of the enzyme to be at pH 5·0, and found very little activity displayed at pH 3·0.

Effect of substrate concentration

α-Mannosidase. The effect of variation in the substrate concentration on the reaction velocity at the pH optimum with *p*-nitrophenyl α-mannoside over the range 0·8–24 mM and phenyl α-mannoside over the range 2·5–40 mM is shown in Fig. 3. In neither case was a maximum velocity reached, but

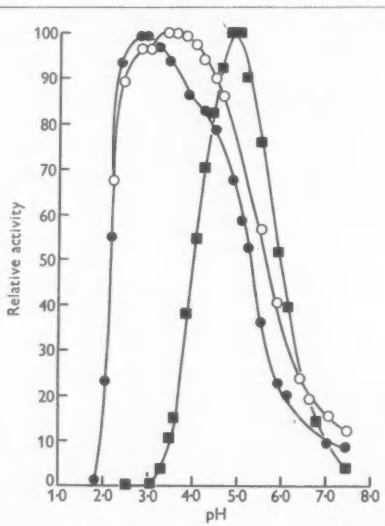

Fig. 1. Hydrolysis at varying pH values of 6 mM-*p*-nitrophenyl α-mannoside in 0·125M-acetic acid–NaOH buffer (■), 2·5 mM-*o*-nitrophenyl β-galactoside in 0·125M-acetic acid–NaOH buffer (●) and 2·5 mM-*o*-nitrophenyl β-galactoside in phosphate–citrate buffer (○), by enzymes from rat epididymis. For other assay conditions see text. An activity of 100 is equivalent to 110 μg. of *p*-nitrophenol and 130 μg. of *o*-nitrophenol liberated/hr.

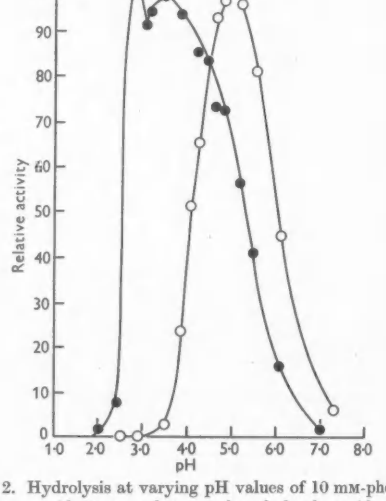

Fig. 2. Hydrolysis at varying pH values of 10 mM-phenyl α-mannoside (○), and 5 mM-phenyl β-galactoside (●), in 0·1M-acetic acid–NaOH buffer by enzymes from rat epididymis. For other assay conditions see text. An activity of 100 is equivalent to 40 μg. (○) and 33 μg. (●) of phenol liberated/hr.

estimations at higher substrate concentrations could not easily be made owing to the limited solubility of the substrates in the incubation mixture. The results were analysed by the method of Lineweaver & Burk (1934), as in Fig. 8, plotting $1/S$ against $1/v$ (S being the substrate concentration and v the reaction velocity). Values obtained for K_m, the dissociation constants of the enzyme–substrate complexes, were 13 mM for *p*-nitrophenyl α-mannoside (mean of seven experiments) and 57 mM for phenyl α-mannoside (mean of three experiments). For routine assays all further estimations were made with the nitrophenyl substrate at a concentration of 6 mM, and the phenyl substrate at a concentration of 10 mM. Most of the experiments described below were carried out with the nitrophenyl substrate only.

β-Galactosidase. Fig. 3 also shows the variation in β-galactosidase activity with concentrations of *o*-nitrophenyl β-galactoside over the range 0·25–12·5 mM and phenyl β-galactoside over the range 0·5–40 mM. Maximal activity was reached with concentrations of 5 mM-*o*-nitrophenyl β-galactoside and 15 mM-phenyl β-galactoside. In each case there was only slight inhibition by excess of substrate. Analysis by the method of Lineweaver & Burk (1934) gave values for K_m of 0·38 mM for *o*-nitrophenyl β-galactoside (mean of 12 experiments), and 1·6 mM for phenyl β-galactoside (mean of three experiments). For routine purposes, the substrate concentrations used were 2·5 mM for the nitrophenyl substrate and 5 mM for the phenyl substrate. Both concentrations lie very near the respective optima for the substrates.

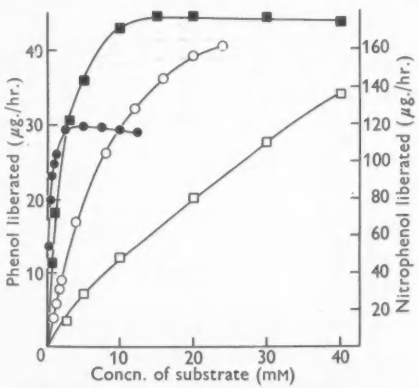

Fig. 3. Effect of varying the substrate concentration on the rate of hydrolysis of *p*-nitrophenyl α-mannoside in 0·125 M-acetic acid–NaOH buffer, pH 5·0 (○), phenyl α-mannoside in 0·1 M-acetic acid–NaOH buffer, pH 5·0 (□), *o*-nitrophenyl β-galactoside in 0·125 M-acetic acid–NaOH buffer, pH 2·9 (●), and phenyl β-galactoside in 0·1 M-acetic acid–NaOH buffer, pH 2·9 (■), by rat-epididymal enzymes. For other conditions of assay see text.

Effect of duration of incubation

Fig. 4 shows the amounts of *p*- and *o*-nitrophenol liberated from 6 mM-*p*-nitrophenyl α-mannoside and 2·5 mM-*o*-nitrophenyl β-galactoside after incubation with enzyme for varying periods. With α-mannosidase, the curve is approximately linear up to a period of 1 hr., after which there appears to be slow enzyme inactivation. The amount of *p*-nitrophenol liberated after 4 hr. represents about 6 % of the total quantity present as glycoside. The rate of hydrolysis of *o*-nitrophenyl β-galactoside was linear with time up to a period of about 3 hr. After 4 hr. about 23 % of the total amount of substrate present was hydrolysed.

Effect of enzyme concentration

The rate of liberation of *p*-nitrophenol from 6 mM-*p*-nitrophenyl α-mannoside was shown to be linear over an eightfold range of enzyme concentration. From this it may be noted that the substrate concentration was sufficiently high to prevent any change in the initial reaction velocity. At the highest concentration of enzyme about 6 % of the total amount of substrate present was hydrolysed.

The rate of hydrolysis of 2·5 mM-*o*-nitrophenyl β-galactoside was linear over a 12-fold range of enzyme concentration. The higher values of liberated *o*-nitrophenol were read after dilution with a buffer mixture corresponding to the final

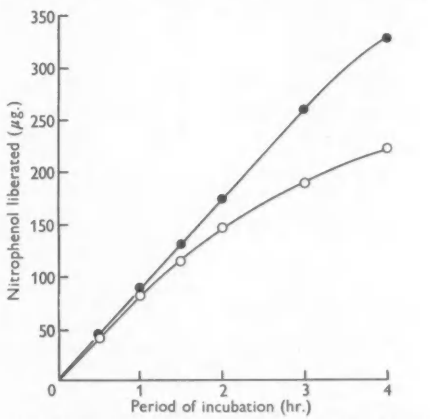

Fig. 4. Effect of incubation at 37° on the hydrolysis of 6 mM-*p*-nitrophenyl α-mannoside in 0·125 M-acetic acid–NaOH buffer, pH 5·0 (○), and 2·5 mM-*o*-nitrophenyl β-galactoside in 0·125 M-acetic acid–NaOH buffer, pH 2·9 (●), by rat-epididymal enzymes.

colorimetric medium. The highest value corresponded to hydrolysis of about 26 % of the original amount of substrate present.

The linear relationship obtained in both cases indicated that there were no natural substrates, activators or inhibitors present in the enzyme preparations.

Effect of temperature

The variation in α-mannosidase and β-galactosidase activities after heating for 5 min. at various temperatures is shown in Fig. 5. The unbuffered enzyme preparation [20–80 % $(NH_4)_2SO_4$ fraction], pH 5·5, was heated at the required temperature for 5 min., cooled in ice and then assayed. Results are expressed as percentage of residual activity, the control being an unheated portion. Under the conditions of the experiment β-galactosidase activity was almost completely destroyed at 70° and α-mannosidase activity at 75°.

Variation in stability with pH

Fig. 6 shows the residual α-mannosidase and β-galactosidase activities in an unbuffered enzyme preparation [20–80% $(NH_4)_2SO_4$ fraction] after incubation at various pH values for 1 hr. at 37°. Portions of the preparation were adjusted to the required pH with dilute HCl or NaOH, incubated at 37° for 1 hr. and, after a pH check, readjusted to the original pH of the preparation (5·5). All portions were made up to a fixed volume and assayed as usual, the results being expressed as a percentage of a control which was not given a preliminary incubation. Both enzymes were stable over the pH range 5·0–6·5, but below pH 5·0 their stabilities were markedly different. α-Mannosidase became very unstable below pH 5·0, and the steep slope of the pH–activity curves in this region (Figs. 1, 2) would appear to be due to the fact that there is considerable inactivation of the enzyme. β-Galactosidase, on the other hand, remained completely stable as far as pH 3·0; below this value the enzyme was very unstable.

Inhibition by the corresponding aldonolactones

The aldonolactones mannono-1 → 4-lactone and galactono-1 → 4-lactone are strong inhibitors of α-mannosidase and β-galactosidase activities respectively (Conchie & Levvy, 1957*a*). Inhibition by these lactones is specific, no cross-inhibitory effects being obtained (Conchie *et al.* 1959*a*).

The effect on α-mannosidase activity of varying concentrations of mannono-1 → 4-lactone over the range 0·5–375 mM is shown in Fig. 7, the substrate used being 6 mM-*p*-nitrophenyl α-mannoside; 50 % inhibition of α-mannosidase activity was obtained with 20 mM-lactone. Determinations of K_i, the dissociation constant of the enzyme–inhibitor complex, were carried out, assays being done in the

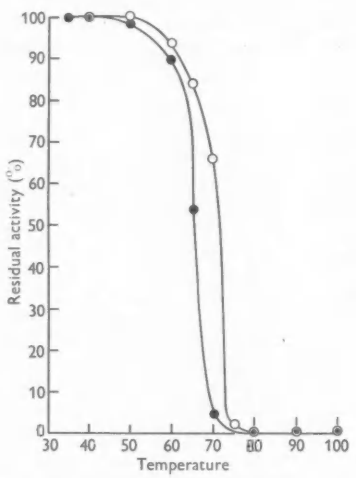

Fig. 5. Effect of heating for 5 min. at varying temperatures on the α-mannosidase (○) and β-galactosidase (●) activities of an unbuffered enzyme preparation from rat epididymis. For conditions of assay see text. Results are expressed as percentage of residual activity, the initial value being that of an unheated control.

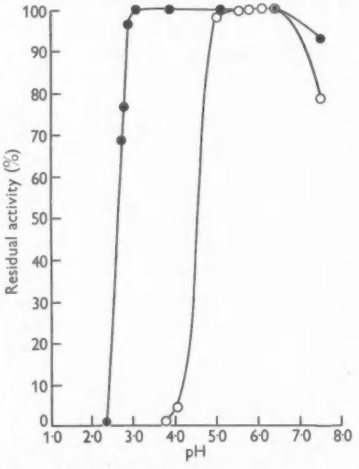

Fig. 6. Effect of incubation for 1 hr. at 37° on the α-mannosidase (○) and β-galactosidase (●) activities of a rat-epididymal enzyme preparation adjusted to varying pH values with HCl or NaOH. For conditions of assay see text. Results are expressed as percentage of residual activity, the initial value being that of an untreated control.

presence and absence of 10 mM-lactone over a substrate concentration range of 0·8–20 mM for *p*-nitrophenyl α-mannoside and 2·5–40 mM for phenyl α-mannoside. Calculation of K_i was made by the method of Lineweaver & Burk (1934), and the analysis of a typical experiment with the nitrophenyl substrate is shown in Fig. 8*a*. Inhibition is competitive, and values for K_m and K_i were 13 and 11 mM respectively. The mean result of six determinations of K_i with the nitrophenyl substrate was 11 mM, and the mean of three determinations with the phenyl substrate was 19 mM. Experiments with the lactone at a concentration of 5 mM with each substrate gave results identical with those already obtained. Thus the inhibitor had an affinity for the enzyme equal to that of *p*-nitrophenyl α-mannoside, and about three times that of phenyl α-mannoside.

Fig. 7 also shows the percentage inhibition of the hydrolysis of 2·5 mM-*o*-nitrophenyl β-galactoside obtained with concentrations of lactones over the range 0·04–40 mM; 50% inhibition was obtained with 2·5 mM-lactone. Determinations of K_i, with *o*-nitrophenyl β-galactoside concentrations over the range 0·5–7·5 mM and lactone at 2 mM, gave an average result of 0·51 mM, the mean of nine experiments. The analysis of a typical experiment by the Lineweaver & Burk plot, giving values of 0·4 and 0·64 mM for K_m and K_i respectively, is given in Fig. 8*b*, which also shows inhibition to be competitive. Measurements made with mM-lactone gave K_i values similar to those already obtained. Determinations of K_i for galactonolactone, with phenyl β-galactoside concentrations over the range 0·5–20 mM and lactone concentrations of 2 and 1 mM, resulted in a mean value of 0·21 mM. The lactone thus had an affinity for the enzyme equal to that of *o*-nitrophenyl β-galactoside and about eight times that of phenyl β-galactoside.

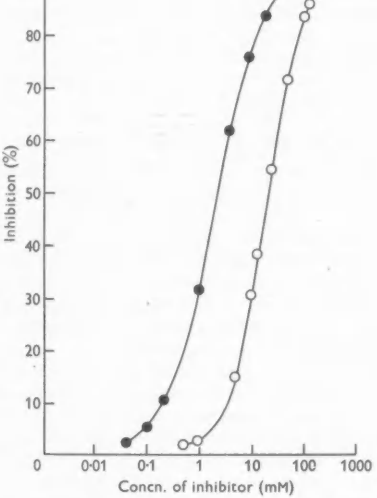

Fig. 7. Inhibition of rat-epididymal α-mannosidase and β-galactosidase activities by varying concentrations of aldonolactones. Mannono-1 → 4-lactone as inhibitor, substrate 6 mM-*p*-nitrophenyl α-mannoside (○); galactono-1 → 4-lactone as inhibitor, substrate 2·5 mM-*o*-nitrophenyl β-galactoside (●). Other assay conditions were as described in the text.

Table 1. *Effects of copper sulphate, silver nitrate and mercuric chloride on α-mannosidase activity after varying periods of pre-incubation with the enzyme*

The substrate was 6 mM-*p*-nitrophenyl α-mannoside in 0·125 M-acetic acid–NaOH buffer, pH 5·0. For 0 hr. values the enzyme was added last to the assay tubes. Results are expressed as percentage inhibition of controls similarly pre-incubated.

		Inhibition (%)			
			Period of pre-incubation (hr.)		
Metal ion	Final concn. (μM)	0 hr.	0·5	1	2
Cu^{2+}	250	25	47	65	84
	500	36	75	84	97
Ag^{+}	10	12	37	48	73
Hg^{2+}	5	45	44	52	64
	10	62	67	77	88

Table 2. *Effects of copper sulphate, silver nitrate and mercuric chloride on β-galactosidase activity after varying periods of pre-incubation with the enzyme*

The substrate was 2·5 mM-*o*-nitrophenyl β-galactoside in 0·125 M-acetic acid–NaOH buffer, pH 2·9. For 0 hr. values the enzyme was added last to the assay tubes. Results are expressed as percentage inhibition of controls similarly pre-incubated.

		Inhibition (%)			
			Period of pre-incubation (hr.)		
Metal ion	Final concn. (μM)	0 hr.	0·5	1	2
Cu^{2+}	500	0	0	7	11
	4 000	5	36	61	86
	10 000	9	82	98	99
Ag^{+}	10	46	47	48	48
Hg^{2+}	0·1	0	1	0	0
	0·2	1	16	7	3
	0·4	23	100	100	100

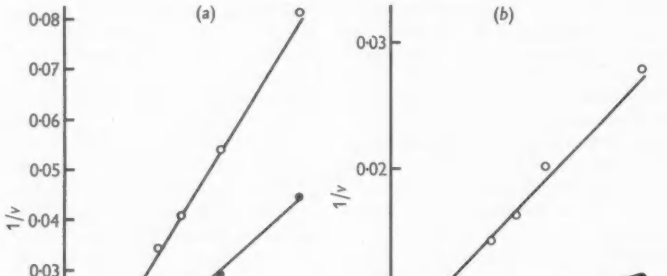

Fig. 8. Relation between S, the substrate concentration (mM), and v, the rate of hydrolysis (μg. of nitrophenol liberated/hr.), by rat-epididymal enzymes in presence (○) and absence (●) of lactone inhibitors, analysed by the method of Lineweaver & Burk (1934), plotting $1/S$ against $1/v$. For conditions of assay see text. (*a*) Substrate, *p*-nitrophenyl α-mannoside; inhibitor, 10 mM-mannono-1 → 4-lactone. (*b*) Substrate, *o*-nitrophenyl β-galactoside; inhibitor, 2 mM-galactono-1 → 4-lactone.

Inhibition by metal ions

The metal ions Ag^+, Hg^{2+} and, in certain cases, Cu^{2+}, have been shown to be powerful inhibitors of β-glucuronidase activity (Doyle, Katzman & Doisy, 1955; Levvy, Hay & Marsh, 1957; Levvy & Marsh, 1957). In view of this, the effects of these ions on the α-mannosidase and β-galactosidase activity of a 20–80% $(NH_4)_2SO_4$ fraction was studied. Tables 1 and 2 show some of the results obtained.

All three ions were strong inhibitors of α-mannosidase activity. The inhibitory effects of Cu^{2+} and Ag^+ ions were considerably increased when the enzyme preparation was pre-incubated at 37° with these ions, the amount of inhibition obtained increasing with the period of pre-incubation. Inhibition by Hg^{2+} ions, on the other hand, was only slightly increased by pre-incubation. Pre-incubations for longer periods were not attempted owing to the considerable inactivation of the enzyme that occurred when it was heated over these periods.

Both Ag^+ and Hg^{2+} ions were powerful inhibitors of β-galactosidase activity. Much higher concentrations of Cu^{2+} ions were required before inhibition was obtained, and this ion was not as effective an inhibitor of β-galactosidase as of α-mannosidase activity. Pre-incubation increased the amount of inhibition obtained with Cu^{2+} and Hg^{2+} ions, but only with Cu^{2+} ions did the length of time of pre-incubation have any effect.

Both enzymes, when incubated alone in their assay buffers over the periods given in Tables 1 and 2, showed much larger falls in activity than were observed when studying the effect of time of incubation on the reaction velocity (Fig. 4). Evidently the presence of substrate stabilizes the enzymes to a considerable extent.

Competing substrate experiments

α-Mannosidase. Investigations were made of the effect of 20 mM-phenyl α-mannoside on the hydrolysis of *p*-nitrophenyl α-mannoside in concentrations over the range 0·2–6 mM. In no case was inhibition of hydrolysis observed. A 40 mM-solution of methyl α-mannoside caused 14% inhibition of the hydrolysis of 6 mM-*p*-nitrophenyl α-mannoside and 19% inhibition of the hydrolysis of 10 mM-phenyl α-mannoside.

β-Galactosidase. Assays were done with *o*-nitrophenyl β-galactoside concentrations over the range 0·5–5 mM in the presence and in the absence of 10 mM-phenyl β-galactoside. Analysis of the results by the method of Lineweaver & Burk (1934) showed inhibition to be competitive, and gave a mean value for K_i for phenyl β-galactoside of 1·9 mM. A similar value was obtained with 5 mM-phenyl β-galactoside. The agreement between this value and the K_m for phenyl β-galactoside already determined, namely 1·6 mM, suggests that the same enzyme is responsible for the hydrolysis of both substrates.

DISCUSSION

The main purpose of the study of the two enzymes was to provide a basis for further investigations, and to compare their properties with each other and with those of the closely associated glycosidases β-glucuronidase and β-*N*-acetylglucosaminidase.

With α-mannosidase, neither of the substrates used could be employed at its optimum concentration owing to the relatively low affinity of the enzyme for the substrates, and limitations of solubility in the incubation mixture. Since the limiting velocity has not been reached, it is particularly important to keep within the limits of aglycone liberation specified (to ensure keeping within the same region of the first-order reaction curve), if results are to have any quantitative value in comparative tissue assays. If the optimum substrate concentrations could conveniently be employed, it is clear that the ultimate activity of α-mannosidase would be much higher relative to other glycosidases than is given in published papers (Conchie & Levvy, 1957*b*; Conchie & Mann, 1957; Conchie *et al.* 1959*a*, *b*; Conchie & Findlay, 1959). The necessary correction factors can be calculated from Fig. 3. Of the two substrates, *p*-nitrophenyl α-mannoside had a higher affinity for the enzyme and has been used for routine assays. The kinetics of β-galactosidase action on the other hand, are fairly simple. Both the chromogenic substrates employed have relatively high affinities for the enzyme, and can be used without difficulty at their optimum concentrations.

Both enzymes are specifically and competitively inhibited by the appropriate aldonolactones. Whereas K_i values for each lactone with the nitrophenyl substrates were not identical with those obtained with the phenyl substrates, they were sufficiently similar to suggest that in each case one enzyme was responsible for the hydrolysis of both nitrophenyl and phenyl substrates. Confirmation was obtained for β-galactosidase by the use of phenyl β-galactoside as a competing substrate in the hydrolysis of *o*-nitrophenyl β-galactoside. The K_i thus obtained for the phenyl substrate was very similar to its K_m. Similar experiments could not be carried out with α-mannosidase, however, since phenyl α-mannoside caused no inhibition of the hydrolysis of *p*-nitrophenyl α-mannoside. No satisfactory explanation of this behaviour can be given, but it seems unlikely that there are present in rat epididymis two separate α-mannosidases with identical pH–activity curves, and which are affected so similarly by mannonolactone and methyl α-mannoside.

Although the glycosidases α-mannosidase, β-galactosidase, β-*N*-acetylglucosaminidase and, to a lesser extent, β-glucuronidase are all closely associated in rat epididymis, it is easy, by use of the appropriate inhibitors, to inhibit one or more of the enzymes as required (Conchie *et al.* 1959*a*). Moreover, concentrations of Cu^{2+} ions can be employed which would inhibit all α-mannosidase activity and leave β-galactosidase activity almost unaffected. From a study of the properties of α-mannosidase and β-galactosidase, it should be possible to obtain a preparation of one enzyme free from any activity of the other. If an enzyme preparation which has been fractionated with ammonium sulphate is incubated for 1 hr. at 37° and pH 3·5, all α-mannosidase activity will be removed whereas β-galactosidase is completely unaffected. On the other hand, heating an unbuffered preparation, pH 5·5, at 70° for 5 min. destroys almost all β-galactosidase activity but only about 33 % of the original α-mannosidase activity.

SUMMARY

1. Some properties of α-mannosidase and β-galactosidase from rat epididymis have been studied, with *p*-nitrophenyl α-mannoside and phenyl α-mannoside as substrates for α-mannosidase and *o*-nitrophenyl β-galactoside and phenyl β-galactoside as substrates for β-galactosidase.

2. The pH optimum of α-mannosidase in various buffers and for both substrates was at 5·0. The pH optimum of β-galactosidase in acetic acid–sodium hydroxide buffer with the nitrophenyl substrate was at 2·9; in citric acid–sodium hydroxide or in phosphate–citrate buffers with the nitrophenyl substrate, and in acetic acid–sodium hydroxide buffer with the phenyl substrate, two optima were obtained, at pH 2·9 and 3·5.

3. Mean K_m values obtained were: *p*-nitrophenyl α-mannoside 13 mM, phenyl α-mannoside 57 mM, *o*-nitrophenyl β-galactoside 0·38 mM and phenyl β-galactoside 1·6 mM.

4. When kept at 37° for 1 hr., α-mannosidase was stable over the pH range 5·0–6·5 and β-galactosidase over the pH range 3·0–7·0.

5. Heating at 70° for 5 min. destroyed β-galactosidase activity, and similar treatment at 75° destroyed α-mannosidase activity. α-Mannosidase was stable at 55–60° for 5 min.

6. Mean K_i values for mannono-1 → 4-lactone, a specific competitive inhibitor of α-mannosidase, were 11 mM with *p*-nitrophenyl α-mannoside as substrate and 19 mM with phenyl α-mannoside as substrate.

7. Mean K_i values for galactono-1 → 4-lactone, a competitive inhibitor of β-galactosidase, were 0·51 mM with *o*-nitrophenyl β-galactoside as substrate and 0·21 mM with phenyl β-galactoside as substrate.

8. Both enzymes are strongly inhibited by Ag^+ and Hg^{2+} ions in low concentrations; Cu^{2+} ions are powerful inhibitors of α-mannosidase activity and moderate inhibitors of β-galactosidase activity.

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The Biological Action of Substances Related to Thyroxine

11. ACETIC ACID ANALOGUES OF THYROXINE AND TRI-IODOTHYRONINE*

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The acetic acid analogues of thyroxine [3:5-di-iodo-4-(4-hydroxy-3:5-di-iodophenoxy)phenylacetic acid (tetraiodothyroacetic acid)] and tri-iodothyronine [3:5-di-iodo-4-(4-hydroxy-3-iodophenoxy)phenylacetic acid (tri-iodothyroacetic acid)] have been shown by Pitt-Rivers (1953) to possess thyroxine-like activity in rats. Thibault & Pitt-Rivers (1955) subsequently demonstrated that both compounds produced an immediate rise in the oxygen consumption of rat-kidney slices and in the basal metabolic rates of thyroidectomized rats. Immediate effects upon the glycolysis and respiration of cells have been reported (Heimberg, Park, Isaacs & Pitt-Rivers, 1955; Alexander & Bisset, 1958), but the immediate response of whole animals has not yet been confirmed by other workers. The possible physiological importance of tetra- and tri-iodothyroacetic acid, however, has been indicated by the chromatographic demonstration of their formation from thyroxine and tri-iodothyronine respectively in rat-kidney preparations (Roche, Michel, Jouan & Wolf, 1955; Albright, Larson, Tomita & Lardy, 1956). In clinical trials both compounds were shown to relieve the symptoms of myxoedema (Lerman & Pitt-Rivers, 1955; Trotter, 1955; Goolden, 1956) and there was some indication that they had a relatively greater effect upon the serum cholesterol than upon the basal metabolic rate.

* Part 10: Wilkinson & Feetham (1955).

Studies of the distribution of tri-iodothyroacetic acid in the tissues and excreta of the rat have been made by Roche, Michel, Jouan & Wolf (1956*a*) and by Larson & Albright (1958), who found that after the administration of small physiological doses the amount excreted as urinary iodide is less than when tri-iodothyronine was similarly used. In view of the possible clinical use of tri- and tetra-iodothyroacetic acid it seemed desirable to extend these studies by comparing the rates of excretion of both compounds with the corresponding iodothyronines. As deiodination processes have been shown to be important factors in the metabolism of the iodothyronines (Wilkinson, Sprott, Bowden & Maclagan, 1954; Wilkinson & Maclagan, 1954), the effect of the deiodination inhibitor, butyl 4-hydroxy-3:5-di-iodobenzoate (Sheahan, Wilkinson & Maclagan, 1951), has also been investigated in rats treated with the acetic acid compounds.

A preliminary report of some of the results has already appeared (Wilkinson, 1958).

MATERIALS AND METHODS

^{131}I-labelled tri-iodothyroacetic acid. 3:5-Di-iodo-4-(4-hydroxyphenoxy)phenylacetic acid (11·1 mg.) (prepared by the method of Wilkinson, 1956) was dissolved at 0° in 2 ml. of methanol–aq. NH_3 soln. (sp.gr. 0·88) (1:1, v/v). A solution of I_2 (5·7 mg.) and $Na^{131}I$ (5 mc) in aq. 50% methanol (4 ml.) was added gradually over 30 min. After setting

aside for 1 hr. the mixture was evaporated under reduced pressure to about 2 ml., the temperature being kept at 0° throughout. The solution was then acidified with HCl and set aside at 0° for a further 1·5 hr., after which the product was collected by centrifuging for 5 min. at 2000 rev./min. The supernatant was removed and the residue washed three times with water (8 ml.). The wet residue was recrystallized from aq. methanol and dried at 20°/1 mm.Hg over $CaCl_2$. Yield: 6·9 mg. The product was shown to be pure by paper chromatography and radioautography. The specific activity was usually in the range 50–100 μc/mg.

^{131}I-labelled tetraiodothyroacetic acid. This was prepared similarly by treating a solution of tri-iodothyroacetic acid (11·2 mg.) in 1 ml. of methanol–aq. NH_3 soln. (sp.gr. 0·88) (1:1, v/v) with a solution of I_2 (4·5 mg.) and $Na^{131}I$ (5 mc) in aq. 50% methanol (2 ml.). The specific activity of the product was 69 μc/mg. For later experiments materials of higher specific activity (e.g. 1 μc/μg.) were prepared by the method of Roche, Michel, Jouan & Wolf (1956*b*), in which the product was separated from inorganic iodide by paper electrophoresis. ^{131}I-labelled thyroxine (specific activity 1 mc/mg.) was purchased from The Radiochemical Centre, Amersham, Bucks. and was diluted with sufficient non-radioactive thyroxine to give convenient counting rates in the animal experiments. ^{131}I-labelled tri-iodothyronine (specific activity 60 μc/mg.) was prepared by the method of Wilkinson & Maclagan (1954).

Excretion studies

Solutions for injection. ^{131}I-labelled tri- or tetra-iodothyroacetic acid (1 mg.) was dissolved in sterile aq. 50% propylene glycol (0·5 ml.) containing diethanolamine (0·05 g.) and the volume made up to 10 ml. with sterile water. The solution was then diluted with sterile 0·9% NaCl soln. so that the required dose was contained in 1 ml. Butyl 4-hydroxy-3:5-di-iodobenzoate (BHDB; 1 g.) was dissolved in 50% propylene glycol (5 ml.) containing diethanolamine (0·3 g.). The volume was made up to 20 ml. with water.

Animals. Male albino rats (100–120 g.) were fed on cubed stock diet and tap water containing 10 mg. of KI/l., both *ad lib.* Groups of three were injected subcutaneously with tri- or tetra-iodothyroacetic acid and kept in metabolism cages, from which the urine and faeces were collected daily. The excreta were treated and the radioactivity was measured as described by Wilkinson *et al.* (1954). In experiments involving the administration of BHDB this was injected subcutaneously (25 mg./rat) on the day preceding the tri- or tetra-iodothyroacetic acid injection and on each subsequent day during the experiment.

Measurements of oxygen consumption

Mice weighing 16±4 g. were randomized into groups of eight. Two groups were injected subcutaneously with tri-iodothyroacetic acid (2 or 5 mg./kg. in 0·02 N-Na_2CO_3) and two with the tetra-iodo compound (2 or 5 mg./kg. in 0·02 N-Na_2CO_3). Two further groups received the medium only and served as controls. Measurements of oxygen consumption were made at intervals of 3–5 hr. over the next 2 days by the closed-circuit method of Maclagan & Sheahan (1950) as modified by Sheahan *et al.* (1951). All results were expressed as a percentage of the controls and their statistical significance was analysed by Student's *t* test.

In order to measure the effect of BHDB on the oxygen consumption of mice treated with tri- or tetra-iodothyroacetic acid, two groups of eight mice were given BHDB (50 mg./kg.) in 0·2 ml. of arachis oil by intraperitoneal injection at 10.30 a.m. and again at 5 p.m. on day 0. These and two further groups of eight mice received single injections of tri- (or tetra-)iodothyroacetic acid (5 mg./kg.) in 0·2 ml. of 0·02 N-Na_2CO_3 at 12.30 p.m. on day 0. The tri- (or tetra-)iodothyroacetic acid controls were given arachis oil at the same times as the BHDB-treated groups received their injections. Two untreated control groups were injected with the media only. Measurements of oxygen consumption were commenced at 11 a.m. on day 1, after which the BHDB-treated animals were given a third injection at 5 p.m. on day 1, and these and the tri- (or tetra-)iodothyroacetic acid controls were treated with a second dose of tri- (or tetra-)iodothyroacetic acid at 4 p.m. on day 1. Measurements of oxygen consumption were repeated on day 2. Since BHDB had not been administered previously in arachis oil, it was confirmed, by a similar experiment in which thyroxine was used in place of tri-iodothyroacetic acid, that it exerted an antithyroxine effect when injected in this medium.

RESULTS

Excretion of tri- and tetra-iodothyroacetic acid in rats

The urinary radioactivity of rats treated with ^{131}I-labelled tri- or tetra-iodothyroacetic acid was shown by paper chromatography and radioautography to consist almost exclusively of iodide. This was confirmed by the fact that addition of serum to the urine followed by protein precipitation with trichloroacetic acid did not significantly reduce the counting rate. Like thyroxine and tri-iodothyronine the acetic acid analogues were co-precipitated with protein. By contrast the faecal radioactivity was substantially removed by co-precipitation with protein and thus appeared to consist of unchanged tri- or tetra-iodothyroacetic acid or conjugates of these. In order to prevent losses of urinary radioiodide due to uptake by the thyroid gland, the animals were given small amounts of iodide in their drinking water.

Fig. 1 shows the result of a typical experiment in which the urinary excretion after the administration of equimolecular doses of tri- and tetra-iodothyroacetic acid were compared. The latter compound was deiodinated to a lesser extent than the former, a finding which proved to be independent of the dose administered. The percentage of the dose excreted as urinary iodide within 72 hr. of administration of various doses of thyroxine, tri-iodothyronine, tri- and tetra-iodothyroacetic acid is shown in Fig. 2. Both acetic acid analogues underwent deiodination to a markedly less extent than the iodothyronines, that of tetraiodothyroacetic acid being significantly less ($P < 0·01$) than that of tri-iodothyroacetic acid.

The faecal output in these experiments is shown in Fig. 3. Except with thyroxine, the percentage

excreted via the intestine also bore no relation to the dose. The rate of excretion of tetraiodothyroacetic acid was significantly lower than those of the iodothyronines, whereas tri-iodothyroacetic acid was excreted more rapidly than any of the other compounds by this route. The combined urinary and faecal excretion (Fig. 4) clearly illustrates the comparatively slow overall rate of excretion of tetraiodothyroacetic acid: only about 50 % of the dose was accounted for in 3 days. Some experiments were therefore continued for a total of 7 days, but even at the end of this time the overall excretion was only 66 %.

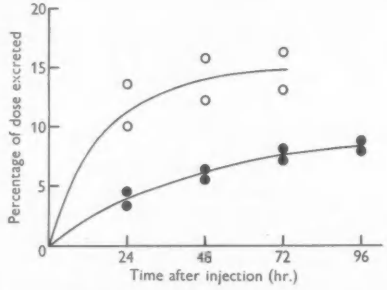

Fig. 1. Excretion of urinary [^{131}I]iodide after subcutaneous injection of equimolecular doses of ^{131}I-labelled tri- and tetra-iodothyroacetic acid in rats. Each point represents the combined excretion of a group of three rats: ○, each rat was given tri-iodoacetic acid (8·3 μg.); ●, each rat was given tetraiodoacetic acid (10 μg.).

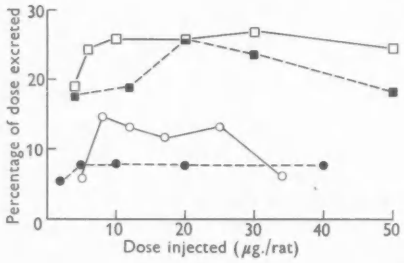

Fig. 2. Rates of excretion of urinary [^{131}I]iodide during the 72 hr. after the subcutaneous injection of ^{131}I-labelled tri- and tetra-iodothyroacetic acid, tri-iodothyronine and thyroxine. Each point represents the mean of two or four observations with the combined excretion of groups of three rats: ○, rats treated with tri-iodothyroacetic acid; ●, rats treated with tetraiodothyroacetic acid; □, rats treated with tri-iodothyronine; ■, rats treated with thyroxine.

Effect of butyl 4-hydroxy-3:5-di-iodobenzoate on deiodination of tri- and tetra-iodothyroacetic acid

The excretion of urinary iodide after the administration of either tri- or tetra-iodothyroacetic acid in rats was markedly reduced by the simultaneous administration of BHDB. Typical results are illustrated in Figs. 5 and 6, but the same effect was observed at several different dose levels.

The effect of BHDB on the radioactivity excreted in the faeces during the 72 hr. immediately after the administration of ^{131}I-labelled tri- and tetra-iodothyroacetic acid, tri-iodothyronine and thyroxine is shown in Table 1. In all four cases an increase occurred, but although this was negligible after the iodothyronines it was especially marked when tetraiodothyroacetic acid was given.

Fig. 3. Rates of excretion of ^{131}I-labelled tri- and tetra-iodothyroacetic acid, tri-iodothyronine and thyroxine in the faeces of rats during the 72 hr. immediately after subcutaneous injection. Each point represents the mean of two or four observations with groups of three rats. See Fig. 2 for explanation of symbols.

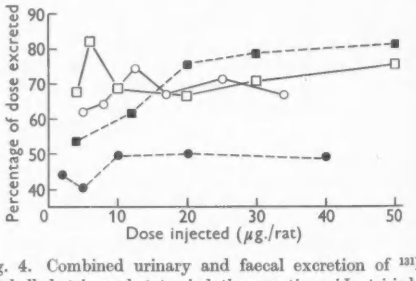

Fig. 4. Combined urinary and faecal excretion of ^{131}I-labelled tri- and tetra-iodothyroacetic acid, tri-iodothyronine and thyroxine in rats during the 72 hr. immediately after subcutaneous injection. Each point represents the mean of two or four observations with groups of three rats. See Fig. 2 for explanation of symbols.

Table 1. *Effect of butyl 4-hydroxy-3:5-di-iodobenzoate on the faecal radioactivity of rats treated with ^{131}I-labelled tri- and tetra-iodothyroacetic acid, tri-iodothyronine and thyroxine*

Compound	Dose (μg./rat)	Percentage of dose appearing in faeces in 72 hr.		
		Rats injected with medium only	Rats injected with BHDB	Mean increase
Tri-iodothyroacetic acid	5	63·7 49·2	72·8 78·7	19·3
Tetraiodothyroacetic acid	2	38·3 39·4	75·2 72·3	34·9
Tri-iodothyronine	6	65·8 49·8	60·3 69·5	7·1
Thyroxine	4	34·5 38·5	54·6 36·1	8·9

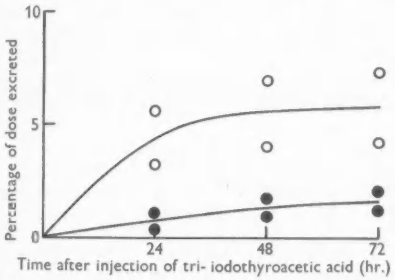

Fig. 5. Effect of BHDB on the excretion of urinary [^{131}I]-iodide in rats injected with ^{131}I-labelled tri-iodothyroacetic acid (5 μg./rat). Each point represents the combined excretion of a group of three rats: ○, given tri-iodothyroacetic acid alone and, ●, in addition given BHDB (25 mg./rat/day).

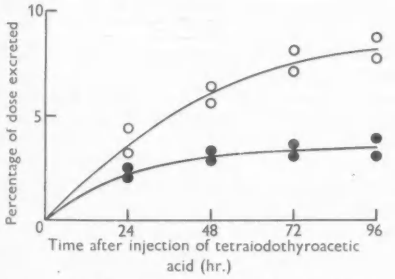

Fig. 6. Effect of BHDB on the excretion of urinary [^{131}I]-iodide in rats injected with ^{131}I-labelled tetraiodothyroacetic acid (10 μg./rat). Each point represents the combined excretion of a group of three rats: ○, given tetraiodothyroacetic acid alone and, ●, in addition given BHDB (25 mg./rat/day).

Effects of tri- and tetra-iodothyroacetic acid on oxygen consumption of mice

Fig. 7 shows the effect of tri- and tetra-iodothyroacetic acid on the oxygen consumption of mice. No effects were observed 4 hr. after the subcutaneous injection of 5 mg./kg., but significant elevation occurred 10 hr. after administration with tri-iodothyroacetic acid but not until 24 hr. with tetra-iodothyroacetic acid. The effect was maximal at about 48 hr. with both substances, but had completely disappeared at 72 hr. Similar results were obtained with doses of 2 mg./kg., but at most time intervals the increase in the oxygen consumption was not statistically significant.

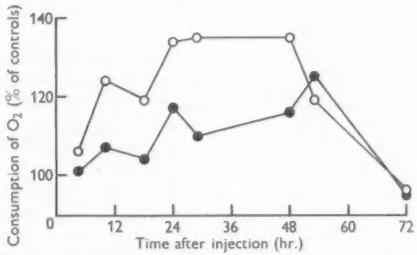

Fig. 7. Effect of tri- and tetra-iodothyroacetic acid upon the oxygen consumption of mice. Each point represents the mean of three readings with each of two groups of eight mice (i.e. six observations): ○, mice injected subcutaneously with tri-iodothyroacetic acid (5 mg./kg.); ●, mice injected subcutaneously with tetraiodothyroacetic acid (5 mg./kg.).

No inhibitory effect was exerted when BHDB was administered at the same time as the iodothyroacetic acids. Thus when expressed as a percentage of the control values the mean oxygen consumption of mice treated with tri-iodothyroacetic acid (5 mg./kg.), 24 hr. after injection, was 123 and 132 in two experiments; the groups treated with BHDB gave figures of 123 and 131 respectively. However, with tetra-iodothyroacetic acid some slight enhancement of the effect was seen, but this was of doubtful significance. The mean oxygen consumption of mice treated with tetra-iodothyroacetic acid was 129 % of the control values in each of two experiments; BHDB-treated animals gave corresponding figures of 138 % ($P < 0{\cdot}05$) and 136 % ($P > 0{\cdot}05$).

DISCUSSION

The finding that the urinary radioactivity of rats treated with ^{131}I-labelled tri- or tetra-iodothyroacetic acid was almost entirely inorganic iodide is consistent with the results of Roche, Michel, Closon & Michel (1957), who, in thyroidectomized rats, found that the principal urinary excretion product of the metabolism of tri-iodothyroacetic acid was inorganic iodide, which, however, was accompanied by several unidentified substances and small amounts of the unaltered acid. During the present study at least 97 % of the urinary radioactivity was accounted for as iodide, and no attempt was therefore made to detect these minor metabolites.

The observation that both acetic acid analogues undergo deiodination *in vivo* to a markedly less extent than the corresponding iodothyronines confirms the finding of Maclagan & Reid (1957), who showed that they were very much less readily attacked by the deiodinating system in rat-liver preparations than tri-iodothyronine and thyroxine. These low rates of deiodination were further depressed when BHDB, the antithyroxine compound, was given at the same time. However, the fact that BHDB had little effect on the oxygen consumption of mice that was enhanced by the treatment with tri-iodothyroacetic acid indicates that deiodination probably does not play an important part in the stimulation of respiration by this substance. The slight enhancement of the tetraiodothyroacetic acid effect by BHDB might be interpreted as indicating that deiodination ordinarily interferes with the respiration-stimulating action of this compound, but the significance of the results obtained is doubtful.

These effects are in marked contrast with those obtained with thyroxine and tri-iodothyronine (Maclagan, Sprott & Wilkinson, 1952), for BHDB antagonized the action of the tetraiodo compound and enhanced that of tri-iodothyronine. It seems that, if the acetic acid compounds appear in the pathway by which thyroxine is degraded, as suggested by Pitt-Rivers (1953), they occur at some stage beyond that at which deiodination has any physiological significance.

The remarkably slow overall rate of excretion of tetraiodothyroacetic acid in comparison with those of tri-iodothyroacetic acid and the iodothyronines was independent of the amount injected, in the range 2–40 μg./rat. The difference in rates of excretion suggested that tetraiodothyroacetic acid might have a more prolonged effect on oxygen consumption than the tri-iodo derivative, but no such effect could be demonstrated. A possible explanation of the slow rate of excretion of the tetraiodo compound has been suggested by Dr J. R. Tata (personal communication), who in electrophoretic studies found it to be more firmly bound to serum protein than tri-iodothyroacetic acid. Alternatively, it may be chemically converted in the body into some inactive substance which is only slowly excreted, a suggestion which is supported by the greater chemical reactivity of this compound (Wilkinson, 1956). The acceleration of the excretion of tetraiodothyroacetic acid by BHDB may be brought about by interference with the formation of this hypothetical inactive compound. Whatever may be the cause of this acceleration of faecal excretion it can be shown, by calculation, that it is not sufficiently great to be solely responsible for the fall in urinary iodide after the administration of BHDB. This view is supported by the studies of Maclagan & Reid (1957), who found that BHDB interfered with the deiodination of both iodothyroacetic acids by liver preparations *in vitro*.

SUMMARY

1. The metabolism of the ^{131}I-labelled acetic acid analogues of thyroxine and tri-iodothyronine has been compared in the rat with that of the corresponding iodothyronines.

2. The urinary radioactivity was shown to consist almost exclusively of iodide and the urinary excretion could therefore be regarded as a measure of the extent of deiodination in all four cases.

3. The acetic acid analogues underwent deiodination to a much smaller extent than the iodothyronines. The overall rate of excretion of tetraiodothyroacetic acid was markedly less than that of any of the other compounds.

4. Both acetic acid analogues increased the rate of oxygen consumption of mice, but no effect was observed until 10 hr. after administration. The duration of this effect was about the same in each case.

5. The antithyroxine compound, butyl 4-hydroxy-3:4-di-iodobenzoate, reduced the degree of deiodination of both acetic acid analogues, but did not diminish the increase in the oxygen consumption produced by these substances in mice.

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A Method for the Estimation of Dehydro*epi*androsterone in Urine

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The evidence indicating the origin of dehydro*epi*androsterone (3β-hydroxyandrost-5-en-17-one) in the adrenal cortex (cf. Lieberman, 1956) has been strengthened by the isolation of this steroid from an adrenal tumour by Plantin, Diczfalusy & Birke (1957). The work of Landau, Knowlton, Lugibihl & Munson (1951), Bitman & Cohen (1951), Dingemanse, Huis in't Veld & Hartogh-Katz (1952) and Ronzoni (1952) suggested that dehydro*epi*androsterone was one of the major 17-oxo steroids in urine. Accordingly its estimation in urine should provide a method of measuring one aspect of adrenal function.

Many methods for the estimation of dehydro*epi*androsterone or of the β-fraction of urine extracts, of which dehydro*epi*androsterone was thought to be the only steroid component, have been described. The earlier methods in which dehydro*epi*androsterone was purified via the digitonide and estimated by the Zimmermann reaction (Talbot, Butler, MacLachlan & Jones, 1940; Baumann & Metzger, 1940; Frame, 1944; Salter, Cahen & Sappington, 1946; Dobriner, 1953) or polarographically (Butt, Henly & Morris, 1948), or methods in which dehydro*epi*androsterone after purification by means of the Girard separation is estimated by the Allen, Hayward & Pinto (1950) reaction (Ronzoni, 1952) or the Pettenkofer reaction (Munson, Jones, McCall & Gallagher, 1948; Landau *et al.* 1951; Bray & Merivale, 1953; Prunty, 1953), or the methods of Talbot, Ryan & Wolfe (1943), Bitman & Cohen (1949, 1951) and Jensen & Totterman (1952), in which dehydro*epi*androsterone is liberated from its conjugate by heating under neutral or slightly acid conditions, lack specificity since no chromatographic separation is used. Fotherby (1958) showed that substances other than dehydro*epi*androsterone, which give positive Pettenkofer and Zimmermann reactions and are precipitated with digitonin, occur in the ketonic fractions of urine extracts. Moreover, most of the earlier methods employ acid hydrolysis at elevated temperatures, which results in the production of artifacts and thus may not be quantitative. In few cases has the sensitivity of the method or the accuracy based upon the amount of added steroid recovered been adequately determined. These remarks also apply to the method described by Cohen & Oneson (1953), in which the steroid sulphates are hydrolysed with dioxan and trichloroacetic acid. Fotherby (unpublished work) found that while pure potassium dehydro*epi*androsterone sulphate is readily hydrolysed by this method the recovery of steroid when the conjugate is added to urine or to urine extracts is poor, presumably owing to the presence of inhibitors of the reaction in the urine extract.

Recently methods for the determination of the major 17-oxo steroids, including dehydro*epi*androsterone, present in urine have been developed, in which either column chromatography (Dingemanse *et al.* 1952; Kellie & Wade, 1957) or paper chromatography (Brooks, 1958; Bush, 1959) is employed, but these methods are lengthy.

It therefore seemed desirable to have a method for the estimation of dehydro*epi*androsterone in

urine that was simple and reliable; the development of such a method, which is described in the present paper, has been made possible by using a procedure for the hydrolysis of the sulphates of the 3β-hydroxy-Δ^5-steroids which appears to be specific for these conjugates.

Talbot *et al.* (1943) and Bitman & Cohen (1949, 1951) showed that the sulphates of 3β-hydroxy-Δ^5-steroids were labile in that they were readily hydrolysed. Thus sodium dehydro*epi*androsterone sulphate could be hydrolysed by heating a solution in acetate buffer (pH 5·8 or 4·7) for 4 hr. at 100°, whereas the sulphates of the saturated 3-hydroxy steroids were not hydrolysed. Teich, Rogers, Lieberman, Engel & Davis (1953) investigated the hydrolysis of sodium dehydro*epi*androsterone sulphate and showed that the products of the hydrolysis depended on the pH of the solution. When a solution of dehydro*epi*androsterone sulphate in a buffer of pH 6·8 was heated under a layer of toluene the major product of the hydrolysis was 6β-hydroxy-3:5-*cyclo*androstan-17-one, accompanied by a small amount of dehydro*epi*androsterone. When the solution was below pH 3, dehydro*epi*androsterone, but no 6β-hydroxy-3:5-*cyclo*androstan-17-one, was isolated, and under these conditions 3β-chloroandrost-5-en-17-one and androsta-3:5-dien-17-one may also be produced. More recently Roy (1956) has shown that, although the sulphates of a number of 3β-hydroxy-Δ^5-steroids are hydrolysed by heating solutions of these compounds at pH 7 at 100°, the sulphates of other steroids are not affected. Thus heating the sulphates of 3β-hydroxy-Δ^5-steroids in approximately neutral solution appears to be a specific method for the hydrolysis of these conjugates.

EXPERIMENTAL

Materials. Ethanol (absolute) was refluxed with KOH pellets and twice distilled. Benzene (A.R.) was redistilled. Acetic acid and sulphuric acid were of A.R. quality. Furfuraldehyde was twice distilled, used immediately to prepare a 0·56% (v/v) soln. in 50% (v/v) aqueous acetic acid, and stored in the refrigerator. Alumina (Hopkin and Williams Ltd., M.F.C., purified, neutral, Brockmann activity = 1) was deactivated by adding water (4 ml./100 g.), stirring to break down moist lumps and shaking for 4–6 hr. The activity was checked by its behaviour with pure dehydro*epi*androsterone. Potassium dehydro*epi*androsterone sulphate was prepared as described by Roy (1956) (Found: C, 56·4; H, 6·6; K, 9·7. Calc.: C, 56·1; H, 6·7; K, 9·6%).

*Method for the estimation of dehydro*epi*androsterone.* For urine from normal humans a 20 ml. sample of a 24 hr. collection was used. The urine was pipetted into a tube with a ground-glass socket and heated without adjustment of the pH under a condenser in a boiling-water bath for 6 hr. (A smaller volume of urine should be used and diluted to 20 ml. with water if the dehydro*epi*androsterone content is expected to be high.) After cooling the urine was extracted with 40 ml. of benzene and the benzene extract washed once with 20 ml. of water. A portion (35 ml.) of the benzene extract was measured into a boiling tube (6 in. × 1 in.) and evaporated to a small volume (about 5 ml.) at 40–60° under a stream of air.

The benzene was then transferred by means of a capillary pipette to a column (6 cm. × 0·5 cm.) of alumina prepared in benzene. The tube which had contained the benzene extract was rinsed with a further 2 ml. of benzene and this was also added to the column. When the benzene had almost passed through the column a further 8 ml. of benzene was added to the column and the benzene eluates were discarded. The dehydro*epi*androsterone and 6β-hydroxy-3:5-*cyclo*androstan-17-one were then eluted from the column with 30 ml. of 0·1% (v/v) ethanol in benzene. This eluate was evaporated to dryness in a warm-water bath under a stream of air, and the residue, together with the standards (20 and 40 μg. of dehydro*epi*androsterone) and a reagent blank, was subjected to the modified Pettenkofer reaction (Munson *et al.* 1948; Fotherby, 1958). The extinctions of the solutions were read at 620, 660 and 700 mμ and the corrected extinction at 660 mμ calculated by using Allen's (1950) correction formula.

RESULTS

*Hydrolysis of potassium dehydro*epi*androsterone sulphate.* A study of the rate of hydrolysis of potassium dehydro*epi*androsterone sulphate in aqueous solution at pH 7 at 100° has shown that the reaction proceeds rapidly at first, approximately 60% of the conjugate being hydrolysed during the first 2 hr., and thereafter decreases in velocity, being approximately 80% complete after 5 hr. and 90% complete after 6 hr. Amounts of potassium dehydro*epi*androsterone sulphate, varying from 5 to 50 μg. in 20 ml. of solution, were used in these experiments, the liberated steroids being extracted with benzene and estimated by the modified Pettenkofer reaction and by the Zimmermann reaction (Callow, Callow & Emmens, 1938).

For the routine procedure a hydrolysis period of 6 hr. was adopted since the hydrolysis is almost complete after this time. The hydrolysis of the dehydro*epi*androsterone sulphate in urine also appears to be complete within 6 hr., for if urine which has been hydrolysed for 6 hr. and extracted is reheated for a further period of 6 hr. and again extracted no dehydro*epi*androsterone is detectable in this second extract.

The evidence at present available (Kellie & Smith, 1957) shows that most, if not all, of the dehydro*epi*androsterone detected in urine is present as the 3β-sulphate, so that heating urine without adjustment of the pH should quantitatively hydrolyse the conjugate of dehydro*epi*androsterone and also the conjugates of the other 3β-hydroxy-Δ^5-steroids present in urine, but should not hydrolyse conjugates of other steroids. Extracts of urine hydrolysed in the above manner are thus less

contaminated with non-steroid material or with other steroids than are extracts of urine hydrolysed by other methods.

It has been shown by paper chromatography with the solvent system light petroleum–methanol–water (5:4:1, by vol.) that approximately equal amounts of dehydro*epi*androsterone and 6β-hydroxy-3:5-*cyclo*androstan-17-one are present in the extracts of urine hydrolysed without acidification. None of the artifact 3β-chloroandrost-5-en-17-one which was isolated by Bitman & Cohen (1951) from extracts of urine hydrolysed by their method was detected; nor could one expect it to be present, since the pH of the urine usually increases by 1–2 units during the hydrolysis, so that acidic conditions which favour the formation of this artifact are not obtained. Also, when potassium dehydro*epi*androsterone sulphate in 20% (w/v) sodium chloride solution or sodium hydroxide solution (pH 10) is hydrolysed by boiling these solutions and the extract is chromatographed on alumina, the amount of material in the dehydro*epi*androsterone fraction reacting positively in the Pettenkofer reaction is equal to that found when an equivalent amount of potassium dehydro*epi*androsterone sulphate is hydrolysed in aqueous solution at pH 7. The rate and extent of hydrolysis of dehydro*epi*androsterone sulphate is not decreased by the presence of sulphate ions in the solution.

*Separation and estimation of dehydro*epi*androsterone and 6β-hydroxy*-3:5-cyclo*androstan-17-one.* The dehydro*epi*androsterone and 6β-hydroxy-3:5-*cyclo*androstan-17-one liberated by the hydrolysis of urine can be purified by chromatography on a column (6 cm. × 0·5 cm.) of alumina. After passing 15 ml. of benzene through the column to remove substances which are present in some urines and interfere in the subsequent colour reaction the dehydro*epi*androsterone and 6β-hydroxy-3:5-*cyclo*androstan-17-one are eluted together by 30 ml. of 0·1% (v/v) ethanol in benzene. Androst-5-ene-3β:17β-diol, which has also been shown to occur in the urine of normal humans (Fotherby, 1958) and of patients with an adrenal tumour (Schiller, Miller, Dorfman, Sevringhaus & McCullagh, 1945; Hirschmann & Hirschmann, 1945; Mason & Kepler, 1945), is not eluted by 50 ml. of 0·1% (v/v) ethanol in benzene.

Variation in the conditions for the Pettenkofer reaction. The effect of varying the conditions under which the reaction is performed has been investigated. As the concentration of the furfuraldehyde was increased the extinction produced from equimolar amounts of dehydro*epi*androsterone was found to increase, but so also did the extinction of the reagent blank. Increasing the molarity of the sulphuric acid used increased the extinction of the reagent blank, and when the molarity of the acid was decreased a much longer time was needed for colour development. A longer time for development was also required when the temperature at which the reaction was performed was decreased. Thus the intensity of colour produced by the incubation of dehydro*epi*androsterone at 45° for 1 hr. was similar to that produced by a 12 min. incubation period at 70°, with 8 M-sulphuric acid and 0·56% (v/v) furfuraldehyde solution. The latter conditions, which are the ones described by Munson *et al.* (1948), have been used in the present method. Under these conditions the reagent blank has very little colour (extinction less than 0·020 at 660 mμ), and because of the short reaction time a large number of estimations can be performed rapidly.

As the length of the incubation period at 70° was increased the extinction at 660 mμ of both the standard solution and the reagent blank increased, whereas the corrected extinction at 660 mμ decreased; after incubation for 1 hr. the corrected extinction had fallen by one-third of the value obtained after a 12 min. incubation period. The colour developed by incubation for 12 min. is stable at room temperature for at least 2 hr.

There is a slight day-to-day variation in the intensity of the colour produced by the standard solution. Although the extinctions of solutions are linearly related to the amount of dehydro*epi*androsterone present, the slope of the line obtained with amounts of dehydro*epi*androsterone greater than 20 μg. is less steep than the slope obtained with amounts of dehydro*epi*androsterone between 0 and 20 μg. For this reason it is advisable to include two standard amounts (20 and 40 μg.) of dehydro*epi*androsterone with each series of estimations. The intensity of colour produced by equal amounts of dehydro*epi*androsterone and 6β-hydroxy-3:5-*cyclo*androstan-17-one is identical. Presumably under the acidic conditions of the Pettenkofer reaction the 6β-hydroxy-3:5-*cyclo*steroid is converted into the 3β-hydroxy-Δ^5-steroid, since this transformation is known to occur in dilute acid solution.

Evaluation of the method. The values found for the excretion of dehydro*epi*androsterone by 28 normal men (ages 18–63 years) range from 0·2 to 4·5 mg./day (mean 1·2 mg.), and for 28 normal females (ages 18–72 years) the range is 0·0–2·0 mg./day (mean 0·5 mg.). These amounts constitute 4–30% and 3–28% respectively of the total 17-oxo steroids. Five patients who have been treated with amounts of adrenocorticotrophic hormone sufficient to produce maximal stimulation of the adrenal have shown increases in dehydro*epi*androsterone excretion varying from three- to twenty-fold compared with the pre-stimulation values.

The recovery of dehydro*epi*androsterone from water or urine to which potassium dehydro*epi*androsterone sulphate has been added is shown in

Table 1. The results shown in Table 1 also give an indication of the sensitivity of the method. The recovery of dehydro*epi*androsterone after the addition of potassium dehydro*epi*androsterone sulphate to water or to urine from adrenalectomized humans is good. The recovery from the urine of normal humans, while acceptable, is a little lower, presumably because of the difficulty in measuring the dehydro*epi*androsterone from the added conjugate in the presence of that originally present in the urine.

By using the formula described by Snedecor (1952) an estimate of the precision of the method can be calculated from the difference between the two results of duplicate determinations in a series of estimations. From duplicate estimations of dehydro*epi*androsterone on forty-five different urine samples the estimate of the precision expressed as the estimate of the standard deviations of the results from the means of the duplicate estimations is 0·06 (calculated in terms of mg./24 hr.).

DISCUSSION

On the evidence available (Lieberman, 1956) dehydro*epi*androsterone appears to originate solely in the adrenal cortex, so that the estimation of this steroid in urine provides a method of measuring one aspect of adrenocortical function. It should be emphasized, however, that the function of dehydro*epi*androsterone and the pathway by which it is produced are at present unknown. This steroid seems nevertheless to be of some importance, for it appears to be the major 17-oxo steroid present in blood (Migeon, 1955; Kellie & Smith, 1957).

The range of values obtained by the method described for the amount of dehydro*epi*androsterone excreted by normal males and females are similar to those reported by Kellie & Wade (1957) and Brooks (1958), who have used methods, which appear to be reliable for the fractionation of urinary 17-oxo steroids, employing the Zimmermann reaction after column or paper chromatography. Thus Kellie & Wade (1957) give values of 0·2–2·5 mg./day and 0·8–2·1 mg./day for six males and six females respectively, while Brooks (1958) found a range of 0·1–1·1 mg./day for six females.

Table 1 shows that the recovery of dehydro*epi*androsterone after adding potassium dehydro*epi*androsterone to water or to urine is good. These recovery figures are a measure of the accuracy of the method, since the dehydro*epi*androsterone added to the urine is in the conjugated form in which it is known to occur in urine. The results shown in Table 1, particularly the recoveries from urine from adrenalectomized humans, indicate also the sensitivity of the method. Thus it is possible to measure accurately 3·4 μg. of dehydro*epi*androsterone added to 20 ml. of urine from adrenalectomized humans, and assuming a daily urine volume of 1500 ml. these values would correspond to an excretion of 0·25 mg. of dehydro*epi*androsterone/day. The sensitivity could be increased by using a larger volume of urine for hydrolysis and extraction or by decreasing the volume of reagents used in the modified Pettenkofer reaction and using micro-cells for the measurement of the extinction.

The results obtained by performing duplicate determinations on a large number of samples of urine show that the reproducibility of the method is good, the estimate of the standard deviation of the results from their means being 0·06 mg./24 hr.

The specificity of the method depends upon the specificity of the hydrolysis procedure, the chromatographic separation of the hydrolysis products and their estimation by the modified Pettenkofer reaction. Munson *et al.* (1948) have shown that this reaction is fairly specific for the 3β-hydroxy-Δ^5-steroids; although the chromogenic character of the members of this group of steroids varies widely, dehydro*epi*androsterone produces one of the most intense colours—a factor which not only aids the sensitivity of the method but also minimizes the interference from other possible compounds that might be expected to be present, e.g. pregnenolone or pregn-5-ene-3β-20α-diol, but which have not yet been demonstrated to occur in urine from normal humans. The method is simple and by its use dehydro*epi*androsterone can be estimated rapidly and accurately.

Owing to the scarcity of a suitable enzyme preparation for the hydrolysis of steroid sulphates in urine, the method commonly used for hydrolysing these conjugates (Lieberman & Dobriner, 1948; Kellie & Wade, 1957; Brooks, 1958) con-

Table 1. *Recovery of dehydroepiandrosterone from water and urine*

In column 3 the results are expressed as ±S.D., with the numbers of estimations in parentheses.

Fluid used	Wt. of potassium dehydro*epi*androsterone sulphate added to 20 ml. of fluid (μg.)	Mean percentage recovery of dehydro*epi*androsterone
Water	4·8	94±5 (7)
	9·6	81±3·4 (7)
	19·2	89±9·0 (11)
	38·4	96±3·2 (5)
Urine from adrenalectomized humans	4·8	97±11 (7)
	9·6	96±5·5 (7)
	19·2	95±6 (6)
	38·4	94±4 (6)
Urine from normal humans	9·6	80±8 (11)
	19·2	79±8 (15)
	38·4	87±10 (12)

sists in continuously extracting urine acidified to pH 1 for 48–72 hr. with ether. Recently Burstein & Lieberman (1958) have described a solvolysis procedure for splitting these conjugates. Although these procedures hydrolyse the sulphates of the 3-hydroxy-5α- and -5β-steroids in addition to the 3β-hydroxy-Δ^5-steroids, the former method has the disadvantage that the hydrolysis is slow and, more important, that more labile steroids, e.g. 3β:16α-dihydroxyandrost-5-en-17-one, are destroyed by these conditions. Whether or not these labile steroids are destroyed by the method of Burstein & Lieberman remains to be determined. The hydrolysis procedure used in the present method is comparatively quick and the more labile steroids appear not to be destroyed, so that the method should be capable of extension to include the other 3β-hydroxy-Δ^5-steroids that have been shown to occur in the urine of normal males (Fotherby, 1958, 1959).

SUMMARY

1. A method for the estimation of dehydro*epi*androsterone in urine is described in which the sulphate of this steroid is hydrolysed by heating urine without acidification at 100° for 6 hr.

2. The criteria of reliability of the method are discussed.

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Diet and the Action of Cortisone on Protein Metabolism

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The general action of administered cortisone is to cause a loss of protein from the body. Some organs, however, do not share in this catabolic effect. In the liver and other viscera, there is an accumulation of protein in response to cortisone administration (see review by Trémolières, Derache & Lowy, 1955). In the experiments to be described below, the influence of diet on these actions of cortisone has been examined. Diets

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varying in both protein and energy content were given to rats and it was found that neither dietary factor affected the general loss of nitrogen from the body which results from cortisone administration. The increase in liver-protein content induced by cortisone was also uninfluenced by the protein content of the diet, but it was found to depend to a considerable degree on an adequate energy intake. This latter finding has been related to the levels of energy-yielding metabolites in the liver on the various diets.

A preliminary report of this work has already appeared (Goodlad & Munro, 1958).

EXPERIMENTAL

Animals. Female albino rats weighing 180–200 g. were housed under thermostatic conditions (23–25°) in individual cages fitted for collection of urine and faeces (Thomson & Munro, 1955). Their body surface areas were computed from the formula of Lee (1929).

Diets and treatment with cortisone. The general dietary arrangements were modelled on those used in previous experiments to provide different levels of protein and of energy intake (Munro & Naismith, 1953). At 9 a.m., 1 g. of a vitamin–mineral–roughage mixture (Munro, 1949) was given to all animals. At 5 p.m. they ate a meal which provided all the protein of the diet. With rats receiving an adequate protein intake, the evening meal contained 2·4 g. of casein (unextracted grade, Glaxo Laboratories Ltd., Greenford, Middlesex), 1·2 g. of carbohydrate (equal parts of glucose and potato starch) and 0·4 g. of fat (margarine). The corresponding evening meal for animals on the lower level of protein intake contained 1·2 g. of casein, the caloric content of the meal being kept constant by increasing the carbohydrate to 2·5 g. For rats receiving the protein-free diet, all the casein of the meal was replaced by an isocaloric amount of carbohydrate, bringing the total carbohydrate content of the meal to 3·8 g.

At each of these three levels of protein intake, the basic diet provided 21 kcal./rat/day and carbohydrate was added to the morning meal to bring the energy intake up to any desired level. At first, all rats were given a total energy intake of 33 kcal./day (about 1200 kcal./sq.m. body surface area) for a period of 5 days, in order that they might become accustomed to the diets. This energy intake was obtained by adding 2·5 g. of starch and 0·5 g. of glucose to the morning meal. After this preliminary period of adjustment, the animals at each level of dietary protein were divided into a group receiving a high-energy intake and a group receiving a low-energy intake. Those on the high-energy intake were given 5 g. of starch and 1 g. of glucose in the morning meal, the total daily energy content of the diet being 45 kcal. (about 1800 kcal./sq.m.). With animals receiving the low-energy intake, the supplementary carbohydrate was withdrawn from the morning meal and they were thus reduced to the 21 kcal. provided by the basic diets (about 850 kcal./sq.m.). At each of these two levels of energy intake, half of the rats were injected subcutaneously with 2·5 mg. of cortisone acetate (Upjohn of England Ltd., London, W. 1) suspended in 0·1 ml. of aq. 0·9% NaCl soln., 1 hr. after each of the two daily meals. The other animals (control series) were injected with 0·1 ml. of aq. 0·9% NaCl soln. at these times. In this way, the animals were divided into 12 groups, comprising cortisone-treated and control animals receiving either a high or a low level of energy intake at each of three levels of dietary protein.

Administration of the various diets and cortisone was continued for 4 days, during which urine and faeces were collected individually from the rats by the method of Thomson & Munro (1955). At the end of the period, the rats were killed in the fasting state by stunning and exsanguination, either at 8 p.m. on the last day of feeding (in which case the meal at 5 p.m. was withheld), or at 10 a.m. on the morning after the end of the 4-day period. The liver was removed for analysis.

Analysis of excreta. The total output of N in the urine and faeces over the period of collection was estimated by the micro-Kjeldahl procedure (Munro & Naismith, 1953).

Analysis of liver. The livers were analysed for protein N, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) by procedures described previously (Munro & Naismith, 1953). The total amounts of these liver constituents have been expressed as mg./100 g. of initial body wt.

The adenine nucleotide content and glycogen content of the livers were estimated in some experiments. Adenosine mono-, di- and tri-phosphate (AMP, ADP and ATP respectively) were measured by the spectrophotometric enzymic assay of Kalckar (1947). Preliminary freezing of the liver was not carried out because Rowles (1952) and Dianzani (1957) obtained higher values for the ATP content of unfrozen tissues. A sample of liver of about 2 g. was rapidly weighed and disintegrated in a homogenizer (Measuring and Scientific Equipment Ltd., London) for 2 min. in 20 ml. of ice-cold 1·5% (w/v) perchloric acid. After removal of the precipitate by centrifuging and washing with 1·5% perchloric acid, a sample (10 ml.) was added to 10 ml. of succinate buffer (Kalckar, 1947) and the pH adjusted to 6·1 by addition of NaOH. A further 15 ml. of buffer was added to the sample and the total volume made up to 50 ml. with water. The three adenosine phosphates were then estimated enzymically on 2·5 ml. samples of this solution, with adenylic deaminase prepared from rabbit muscle (Kalckar, 1947), commercial myokinase (Boehringer und Soehne, Mannheim) and apyrase prepared from potatoes (Krishnan, 1949).

RESULTS

Our results represent the action of cortisone over a 4-day period at different levels of protein intake, on each of which energy intake was also varied.

Effect of cortisone on body weight and liver weight. A number of investigators have demonstrated in short-term experiments that cortisone administration causes loss of body weight accompanied by enlargement of the liver (Kochakian & Robertson, 1951; Silber & Porter, 1953; Bodansky & Money, 1954; Trémolières, Derache & Griffaton, 1955; Clark & Pesch, 1956). Most of this information was obtained under conditions of unrestricted feeding. Our data (Table 1) confirm these actions of cortisone under conditions of controlled food intake and show that they are independent of diet. The body weights and liver weights in the control series of

Table 1. *Effect of cortisone administration over a 4-day period on the body weight and liver weight of rats receiving diets varying in protein and energy content*

After a preliminary period on the diet fed at an energy level of 33 kcal./day, the rats at each protein level were divided into a group on a low-energy intake (21 kcal./day) and a group on a high-energy intake (45 kcal./day). Rats in each group were injected with 2·5 mg. of cortisone acetate twice daily for 4 days; control animals received an injection of aq. 0·9% NaCl soln. They were killed on the morning after the end of the 4-day period. Results are the means of seven experiments ±S.E. Statistical analysis shows that the change in body weight over the 4-day period and the final liver weight were affected significantly ($P < 0·01$) by dietary protein level, by dietary energy level and by cortisone administration; the magnitude of the change produced by cortisone was not significantly affected by diet in either case ($P > 0·05$ for interaction of cortisone with dietary protein and energy intakes).

		Initial body wt.		Change in body wt.		Liver wt./100 g. of initial body wt.	
Dietary protein	Dietary energy	Control series (g.)	Cortisone series (g.)	Control series (g.)	Cortisone series (g.)	Control series (g.)	Cortisone series (g.)
None	Low	175	173	−14±5	−16±3	2·27±0·23	2·98±0·13
	High	181	182	−3±2	−17±3	2·65±0·10	3·26±0·13
Low	Low	173	173	−12±3	−21±3	2·54±0·08	2·84±0·07
	High	176	181	+4±1	−7±2	2·67±0·27	3·70±0·22
Adequate	Low	174	175	−10±2	−15±2	2·78±0·14	3·40±0·20
	High	169	164	+3±3	−5±3	2·98±0·11	3·58±0·17

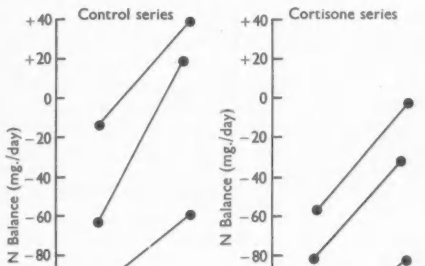

Fig. 1. Effect of cortisone administration over a 4-day period on the nitrogen balance of rats receiving diets varying in protein and in energy content. Experimental conditions were similar to those described in Table 1. Each graph shows the influence of energy intake on nitrogen balance at an adequate level of dietary protein (upper line), at a low level of dietary protein (middle line) and on a protein-free diet (lower line). Results are the means of five experiments. Statistical analysis shows that nitrogen balance was affected significantly ($P < 0·01$) by protein intake, by energy intake and by cortisone administration. The action of cortisone on nitrogen balance was not significantly altered by diet ($P > 0·05$ for the interaction of cortisone with dietary energy and protein intakes).

rats were both considerably affected by the protein and energy content of the diet fed during the 4-day period, but the loss of body weight and gain in liver weight resulting from cortisone administration were not significantly different on the various diets.

Effect of cortisone on nitrogen balance. In agreement with previous evidence (Munro, 1951), N balance was affected by both the protein content of the diet and its energy content (Fig. 1). Comparison of the N balances of the cortisone-treated groups with those of the control groups receiving corresponding levels of protein and energy in the diet shows that cortisone administration caused an increased loss of N from the body on all diets. This action of cortisone was of similar magnitude at all levels of protein and energy intake. In consequence, the improvement in N balance after an increase in protein intake or in energy intake was the same in the cortisone-treated and control series of animals (Fig. 1). Thus cortisone does not interfere with the capacity of the body as a whole to utilize dietary protein or energy.

Effect of cortisone on the protein and nucleic acid content of the liver. The total protein and RNA content of the liver is influenced by the protein and energy content of the diet (Munro & Naismith, 1953; Munro, Naismith & Wikramanayake, 1953). Our present series of experiments confirm these effects of diet on liver composition (Table 2). Cortisone administration increased the amount of protein and of RNA. This increment was independent of the dietary protein level, but it was influenced by energy intake to a significant degree with liver-protein deposition and probably also with RNA accumulation. At each dietary protein level, the rats receiving the higher-energy intake responded to cortisone with a greater deposition of protein and of RNA in the liver, the average deposition over the three protein levels being about twice that of animals fed on diets of low-energy content. Thus the catabolic action of cortisone on the body as a whole is independent of diet, whereas

Table 2. *Effect of cortisone administration over a 4-day period on the protein and ribonucleic acid content of the livers of rats receiving diets varying in protein and in energy content*

Experimental conditions were similar to those described in Table 1. Results are the means of seven experiments ±S.E. Analysis of variance shows that cortisone administration caused a significant deposition of protein in the liver at both levels of energy intake ($P < 0.01$), but the action of cortisone was significantly greater at the higher-energy level ($P < 0.05$ for interaction between cortisone and energy); the action of cortisone was not significantly modified by dietary protein level ($P > 0.05$ for this interaction). The action of cortisone on liver RNA content was only significant at the higher level of energy intake ($P < 0.01$). This influence of dietary energy level on the RNA deposition caused by cortisone administration is also suggested by a tendency for the interaction between energy intake and cortisone to be positive (P almost 0.05).

		Liver protein N (mg./100 g. of initial body wt.)			Liver RNA P (mg./100 g. of initial body wt.)		
Dietary protein	Dietary energy	Control series	Cortisone series	Difference	Control series	Cortisone series	Difference
None	Low	52·3±3·3	62·1±2·5	+9·7	2·26±0·12	2·44±0·08	+0·18
	High	49·9±2·1	67·0±4·2	+17·1	2·43±0·16	2·75±0·16	+0·32
Low	Low	57·8±1·7	58·7±2·0	+0·9	2·32±0·17	2·33±0·09	+0·01
	High	63·7±2·2	75·7±4·1	+12·0	2·65±0·15	3·10±0·18	+0·45
Adequate	Low	64·2±2·1	73·7±3·5	+9·5	2·59±0·16	2·82±0·17	+0·23
	High	71·3±3·1	84·3±4·4	+13·0	2·75±0·15	3·37±0·15	+0·62

Table 3. *Influence of diet and of cortisone on the content of adenosine phosphates in rat liver*

Diets were given as two meals, at 9 a.m. and 5 p.m., the energy content of the diet being varied by addition of carbohydrate to the morning meal and the protein content by changes in the evening meal. After 5 days at a constant energy intake, rats at each protein level were divided into groups on low and on high intakes of energy and were injected with cortisone or aq. 0·9% NaCl soln. (control group) 1 hr. after each meal. They were killed either at 8 p.m. on the fourth day of cortisone treatment (the evening meal being omitted on that day), or at 10 a.m. on the morning of the next day (the morning meal being omitted). The results given are the means of three experiments at each time of killing ±S.E.; the factors affecting them have been evaluated statistically by analysis of variance as ++ ($P < 0.01$), + ($P < 0.05$) or - ($P > 0.05$). There were no significant interactions, which indicates that the concentrations of adenosine phosphates in the liver were influenced by diet to the same extent in the cortisone-treated and control groups.

Treatment	Dietary protein	Dietary energy	AMP (μmole/g.)	ADP (μmole/g.)	ATP (μmole/g.)
		(*a*) Animals killed in the evening			
None	None	Low	1·72±0·19	0·92±0·23	0·43±0·17
		High	1·41±0·06	1·05±0·07	1·28±0·14
	Adequate	Low	1·70±0·22	1·41±0·04	0·61±0·11
		High	1·20±0·18	0·83±0·04	1·50±0·33
Cortisone	None	Low	1·28±0·08	1·40±0·05	1·47±0·32
		High	1·09±0·12	1·14±0·06	1·97±0·32
	Adequate	Low	1·34±0·19	1·38±0·14	1·45±0·22
		High	1·18±0·13	1·23±0·08	1·54±0·28
Statistical analysis:					
Protein			-	-	-
Energy			++	+	++
Cortisone			++	++	++
		(*b*) Animals killed in the morning			
None	None	Low	1·38±0·10	1·23±0·05	0·65±0·17
		High	1·13±0·23	1·02±0·17	1·02±0·04
	Adequate	Low	1·61±0·14	1·16±0·02	0·90±0·05
		High	1·75±0·07	1·20±0·17	0·60±0·10
Cortisone	None	Low	0·91±0·05	1·06±0·13	1·15±0·07
		High	1·19±0·06	1·06±0·15	1·19±0·12
	Adequate	Low	1·21±0·09	1·44±0·20	1·33±0·02
		High	1·09±0·16	1·06±0·33	1·39±0·23
Statistical analysis:					
Protein			+	-	-
Energy			-	-	-
Cortisone			++	-	++

the anabolic effect of cortisone on the liver is partly dependent on energy intake.

Measurement of the total DNA content of the liver (not recorded in Table 2) showed it to be uninfluenced by any of the dietary conditions or by cortisone administration. Silber & Porter (1953) also found no effect of cortisone on the DNA content of liver.

Action of cortisone in relation to the content of adenosine phosphates in the liver. Since our results show that the amount of protein deposited in the liver after cortisone administration depends on energy intake, it was of interest to know whether this finding could be related to the concentrations of adenosine phosphates in the liver at different levels of energy intake. The diets fed to our animals were given in the form of two meals, differences in energy intake being obtained by changing the amount of carbohydrate in the morning meal and differences in protein intake by varying the composition of the evening meal. In order to assess the effect of diet and of cortisone on the adenosine phosphate content of the liver throughout the day, it was therefore necessary to kill animals at definite times after each of these meals. One series of rats was killed at 8 p.m. on the fourth day of cortisone treatment, i.e. 11 hr. after the morning meal; a second series was killed the following morning at 10 a.m., 17 hr. after the evening meal. At both of these times of killing, the cortisone-treated animals differed from the corresponding animals of the control series by having a lower concentration of AMP and a higher concentration of ATP in their livers (Table 3). In the rats killed in the evening, the concentrations of these metabolites were influenced in addition by the energy content of the morning meal, animals on the higher level of energy intake having lower concentrations of AMP and ADP, and higher concentrations of ATP. This effect of level of energy intake was not significantly reduced by cortisone treatment. In the animals killed in the morning, however, the energy content of the diet no longer had a significant effect on the adenosine phosphate content of the liver.

Our data thus show that, for part of each day, animals on the higher level of energy intake have more ATP in their livers, and that cortisone administration does not obliterate this effect of dietary energy level.

DISCUSSION

In a preceding series of experiments (Munro, Black & Thomson, 1959) evidence was obtained of a reciprocal relationship between protein metabolism in muscle and in the viscera. After administration of glucose to fasting rats, there was an increased uptake of labelled amino acids by muscle, and this was accompanied by a fall in the urinary excretion of nitrogen and in the protein content of the liver. It was concluded that the primary action of glucose on protein metabolism is to cause deposition of amino acids in muscle. This curtails the supply of amino acids to other tissues, and in consequence there is a reduction in protein synthesis by these organs and in urea formation. The action of cortisone on protein metabolism is the converse of this picture. Administration of cortisone over short periods results in an increased excretion of nitrogen due to loss of protein from the carcass (Kochakian & Robertson, 1951; Clark, 1953; Silber & Porter, 1953). This is accompanied by an increase in the protein content of the liver and other viscera (Kochakian & Robertson, 1951; Clark, 1953; Silber & Porter, 1953; Bodansky & Money, 1954; Trémolières, Derache & Griffaton, 1955; Clark & Pesch, 1956; Fritz, 1956). As with administered glucose, these metabolic actions of cortisone can be interpreted in terms of a reciprocal relationship between protein metabolism in the carcass and in the viscera. The wasting of carcass protein caused by cortisone provides a greater supply of amino acids for other organs and in consequence protein synthesis in these organs and urea formation are increased. This interpretation gains strength from the fact that other agents causing loss of carcass protein also increase the protein content of the viscera. Deposition of protein in the liver has been observed after administration of thyroid preparations to rats (Sternheimer, 1939; Addis, Karnofsky, Lew & Poo, 1938; Guggenheim, Halevy, Singer & Usieli, 1958), and it also accompanies wasting of the carcass in tumour-bearing rats (Stewart & Begg, 1953), although in the latter case the changes in the liver differ in several respects from those produced by cortisone administration (Goodlad & Munro, unpublished data).

The present experiment were undertaken to determine whether the actions of cortisone on protein metabolism are influenced by diet. Silber & Porter (1953) noted that administration of cortisone increased the urinary output of nitrogen and the protein content of the liver independently of dietary protein intake. Our data show that the catabolic action of cortisone on the body as a whole is independent of the protein and energy content of the diet (Fig. 1), whereas the anabolic action of cortisone on the liver is partly dependent on energy intake (Table 2). The latter finding suggests that the capacity of the liver to utilize amino acids coming from carcass breakdown may be influenced by the energy available in the liver. Direct examination of energy-yielding compounds in the livers of our animals (Table 3) showed that the energy

content of the diet had a considerable effect on the concentrations of AMP and of ATP during part of the day. Although cortisone administration also produced changes in the concentrations of AMP and of ATP, the influence of dietary energy intake was not significantly diminished among such animals. This shows that, in spite of the considerable deposition of glycogen in the liver which is known to result from cortisone administration, the energy content of the diet continued to influence energy metabolism in the livers of the hormone-treated group, and may thus have affected utilization of amino acids arising from carcass breakdown.

Changes in the RNA content of the liver after cortisone administration are consistent with an increased supply of amino acids coming to the liver. A number of authors (e.g. Munro *et al.* 1953) have shown that, when the protein content of the diet is raised, there is an increase in the amount of RNA as well as of protein in the liver. If cortisone administration increases the protein content of the liver because it augments the supply of amino acids coming from the carcass, the RNA content of the liver would be expected to respond in the same way as it does to an increased supply of amino acids from the diet. Silber & Porter (1953) recorded a substantial increase in the liver RNA of cortisone-treated animals, but Lowe & Rand (1956), using much larger doses of the hormone, did not obtain any appreciable increment. In our own experiments, cortisone administration increased the RNA content of the liver (Table 2). As with protein deposition, there was a tendency for the increase in RNA to be greater when the diet provided an abundant supply of energy.

SUMMARY

1. The effect of cortisone on nitrogen balance and on liver protein and ribonucleic acid content has been studied on rats receiving diets varying in protein and in energy content.

2. The catabolic action of cortisone on the body as a whole was independent of the protein and energy content of the diet, whereas the anabolic action of cortisone on the liver was greater when the dietary energy intake was high.

3. The adenosine phosphate content of the liver was affected by the level of energy in the diet, even in the cortisone-treated series of animals.

4. It has been concluded that loss of protein from the carcass after cortisone administration results in a greater supply of amino acids for other organs and in consequence protein synthesis in these organs and urea formation are increased. The capacity of the liver to utilize amino acids arising from the carcass is probably dependent on the supply of energy available in the liver cell.

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The Formation of Thyroid Hormones *in vivo* after the Administration of $K^{131}I$ and Thyrotropic Hormone

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Previous work upon the incorporation of intravenously injected radioactive ^{131}I into the thyroid hormones of the blood, though extensive, has been somewhat restricted since in most instances separation of the individual thyroid hormones has either not been achieved or separation was made upon samples of blood withdrawn at a single interval after injection.

For example, in studying the incorporation of ^{131}I into the serum thyroid hormones in patients suffering from hyperthyroidism and myxoedema and in the normal controls, Robbins, Rall, Becker & Rawson (1952) and Chesky, Dreese, Duboczky, Hall & Hellwig (1953) determined only the total inorganic iodine and total hormonal iodine in the blood. On the other hand, Gross & Pitt-Rivers (1951) determined the quantity and radioactivity of labelled thyroxine and tri-iodothyronine in serum from goitrous patients 24 hr. after injection of radioactive iodine. Many similar studies have been made [for reviews see Gross & Pitt-Rivers (1953) and Roche & Michel (1955)], but in only a few (cf. Taurog & Chaikoff, 1947; Tong, Taurog & Chaikoff, 1951) have attempts been made to measure the relative rates of formation of the various hormones, largely owing to the absence of sensitive methods for the separation and estimation of microquantities of iodine-containing hormones in the blood. Recently (Feuer, 1957; Szabó, Feuer & Balogh, 1957) a micromethod has been developed which permits the quantitative separation and determination of microquantities of thyroxine, tri-iodothyronine and other iodine-containing compounds in the blood and the method has been applied to the study of the rates of formation of thyroid hormones *in vivo* by measurements over a period of time after injection of ^{131}I.

Since the rate of release conditioned by various types of stress is similar, in depletion of ^{131}I of thyroid gland, to that brought about by thyrotropic hormone (Brown-Grant, Euler, Harris & Reichlin, 1954; Brown-Grant, Harris & Reichlin, 1954; Harris, 1955), the rate of release of individual hormones in animals given thyrotropin was also studied.

It has been found that the incorporation of ^{131}I into tri-iodothyronine precedes the incorporation into thyroxine and that thyrotropin accelerates the conversion of tri-iodothyronine into thyroxine.

METHODS

Animals. Rats (200–300 g.) and rabbits (2·5–4·0 kg.) from the Laboratory stock were used in the individual experiments.

Administration of radioactive iodine and thyrotropic hormone. Radioiodine was obtained as carrier-free $K^{131}I$ from The Radiochemical Centre, Amersham, Bucks, and, after dilution with 0·9% NaCl, 0·2–0·5 ml. containing 100 μc of ^{131}I was injected into rats either through the tail vein or, under ether anaesthesia, intraperitoneally. Thyrotropic hormone (Ambinon-B Organon; 50 Junkmann–Schoeller units/ml.) was also given intraperitoneally. Rats received 0·05–0·1 ml. and rabbits received 0·5–1·0 ml. During the experiments the animals were kept in the laboratory at room temperature and were maintained on the normal diet.

At differing times after the administration of ^{131}I or thyrotropic hormone, the rats were anaesthetized and blood was removed through the dorsal aorta with the aid of a syringe. After standing for 3–4 hr. at room temperature the clotted blood was centrifuged and the serum was removed. The serum of one rat, or the combined sera of two rats (10–14 ml.) was used for fractionation of the thyroid hormones. Since the removal of this amount of blood from the rats resulted in death, separate groups of rats were used to establish each point upon the time curves. With rabbits the thyrotropic hormone was given intraperitoneally, the blood samples being taken from the marginal veins of the ears after heparinization of the animals with 1–2 ml. of heparin (Roche Products Ltd., 1000 units/ml.). Samples were taken immediately before the injection of the thyrotropic hormone and at varying times thereafter. The same animal was used to yield all the blood samples required during any one experiment.

Extraction of the thyroid hormones. Serum (3–5 ml.) was brought to about pH 3 with N-HCl and butanol (2 vol.) was added. After shaking vigorously for 30 min. the butanolic phase was separated by centrifuging. This extraction was repeated three times with butanol (1 vol.). The combined butanol extracts were reduced to dryness under diminished pressure below 45°, and the residue was suspended in a volume of water equivalent to one-tenth of the volume of the serum originally taken.

Separation and determination of the thyroid hormones. These were separated by chromatography on paper and the fractions estimated by methods previously described (Feuer, 1957; Szabó *et al.* 1957). Briefly, 0·1 ml. of the aqueous hormonal solution was measured on to paper

(Schleicher and Schüll no. 2043 or Whatman no. 1) and developed by the ascending technique for 18–20 hr. at room temperature with the organic phase from dioxan–butanol–aq. 2N-NH_3 soln. (1:4:5, by vol.). Samples of tri-iodothyronine and thyroxine were included in a guide strip. After development, the hormones in the guide strip were located with ninhydrin and the corresponding adjacent sections were cut from the rest of the chromatogram. These were incinerated at 450–500° in the presence of 0·5 ml. of 2N-Na_2CO_3, the ash was brought to neutrality with N-HCl and the volume adjusted to 3 ml. with water. To a portion was added: NaCl (0·8 g.), 5 mM-ferroin indicator (0·3 ml.) 0·2N-H_3AsO_3 (1·9–2 ml.) and water to 3·5 ml. The solutions were brought to 25°, 0·01N-$Ce(SO_4)_2$ (1·5 ml.) was added and the time taken for the colour to change from blue to red–brown was noted. The end point is fairly sharp, and the period between the addition of $Ce(SO_4)_2$ and the colour change bears a quantitative relationship to the amount of iodine present over the range 2×10^{-4} to 2×10^{-2} μg. of iodine.

Fig. 1 illustrates how the method can be used with a human thyroid sample. The upper part of the figure shows the separation of the concentrated thyroid extract ($1a_1$) and the thyroxine and tri-iodothyronine controls ($1a_2$) on the paper chromatogram; the lower part ($1b$) gives the iodine contents of the various parts of the paper strip.

Determination of the iodine content of the whole blood. After the blood was taken, samples (0·5 ml.) were evaporated and incinerated in the presence of 0·5 ml. of 2N-Na_2CO_3 and the inorganic iodide was measured as described above.

Determination of the protein-bound iodine. The blood was kept at room temperature for 3–4 hr. and, after clotting, the serum was separated by centrifuging. The proteins were precipitated from 0·5 ml. of serum with the $ZnSO_4$–NaOH precipitant of Somogyi. The precipitate was incinerated in the presence of 0·5 ml. of 2N-Na_2CO_3 and the inorganic iodide was measured as described above.

Measurement of radioactivity in the separated fractions. The distribution of radioactivity in the hormonal fractions, after separation by paper chromatography and location with ninhydrin, was determined by sectioning a strip 3 cm. wide at intervals of 1 cm. and measuring the radioactivity with an end-window counter. Samples were counted twice, to an accuracy of 5%.

RESULTS

Incorporation of $K^{131}I$ *into thyroid hormones of the serum*

The incorporation of ^{131}I was studied in albino rats. Fig. 2 represents the results obtained in a typical experiment in which the radioactive iodide was administered to rats 8 hr. before blood was withdrawn. The upper part of the figure shows the position of the ninhydrin-reacting substances found on the paper chromatogram of a serum extract ($2a_1$) compared with known thyroid

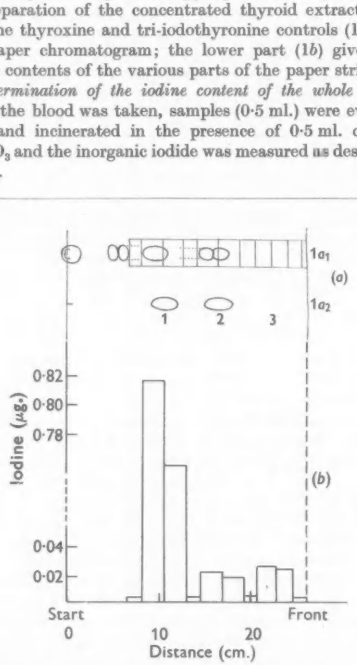

Fig. 1. Separation and determination of thyroid hormones from human thyroid-gland extract. (*a*) Paper chromatogram: $1a_1$, 0·1 ml. of tenfold-concentrated human thyroid-gland extract; $1a_2$, 25 μg. of thyroxine and 25 μg. of tri-iodothyronine; developed by the ascending technique at room temperature with dioxan–butanol–aq. 2N-NH_3 soln. (1:4:5, by vol.) and located with ninhydrin. (*b*) Measurement of iodine content of the thyroid extract: 1, thyroxine, 2, tri-iodothyronine; 3, compound 3.

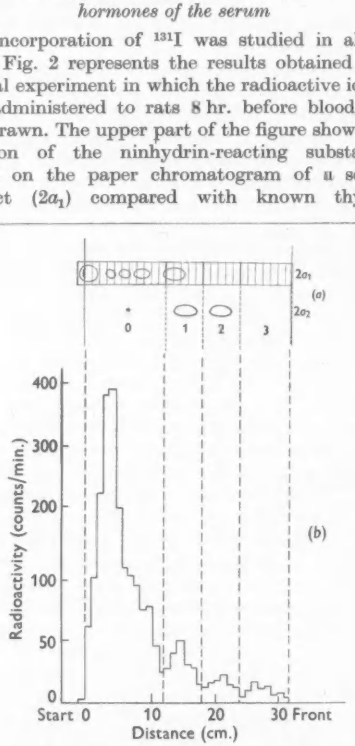

Fig. 2. Incorporation of $K^{131}I$ into thyroid hormones. (*a*) Paper chromatogram: $2a_1$, 0·1 ml. of tenfold-concentrated rat-serum extract; $2a_2$, 25 μg. of thyroxine and 25 μg. of tri-iodothyronine controls; developed by the ascending technique at room temperature with dioxan–butanol–aq. 2N-NH_3 soln. (1:4:5, by vol.) and developed with ninhydrin. (*b*) Measurement of radioactivity of the serum extract: 0, inorganic iodine; 1, thyroxine; 2, tri-iodothyronine; 3, compound 3.

hormones added as markers ($2a_2$). The concentration of the ninhydrin-reacting compounds in the serum extract is very low. The lower part of the figure (*b*) shows the distribution of radioactivity in the chromatogram prepared from the serum extract.

It is seen that the spots corresponding to thyroxine and tri-iodothyronine developed with ninhydrin also correspond to areas of increased radioactivity. Thyroxine: R_F 0·45 (ninhydrin), 0·48 (activity); tri-iodothyronine: R_F 0·61 (ninhydrin), 0·645 (activity). The site of thyroxine was also checked with the aid of [^{131}I]thyroxine. In addition to these compounds, increased radioactivity was also detected at a point on the chromatogram ahead of tri-iodothyronine, indicating the existence of another iodine-containing compound, designated compound 3. The existence of such a compound has been previously demonstrated by Feuer & Vekerdy (1958). Several other authors have described unknown iodine-containing compounds with a high R_F value in the extracts of serum and thyroid of different animals (Tong *et al.* 1951; Vannotti, Cruchaud, Frei & Mahaim, 1955; Taurog, Potter & Chaikoff, 1955; Lissitzky & Roques, 1956; Taurog, Tong & Chaikoff, 1957).

The pattern of incorporation of radioactive iodine into the thyroid hormones of the blood at varying times after the administration of ^{131}I is presented in Fig. 3. In the graph only the organic ^{131}I values are shown. The total amount of this iodine value was taken to be 100 % and the relative amounts of thyroxine, tri-iodothyronine and compound 3 were calculated.

It can be seen that the radioactivity of the thyroxine, which accounted for only 20 % of the total activity of organic iodides at the beginning, increased markedly with time. This increase was fairly rapid in the first 8 hr., becoming slower later, and after 24–48 hr. a steady value of 70–80 % was reached. The relative amount of the radioactive tri-iodothyronine was highest (70–75 %) in the first 1–1½ hr. after the administration of ^{131}I. The activity diminished with a rapidity almost equal to the rise in radioactivity of thyroxine. Later this decrease became slower and after 24–48 hr. an equilibrium value of 10–20 % of the total was reached. The radioactivity of compound 3 was initially smaller than the radioactivity of thyroxine; it increased a little in 6–8 hr., and subsequently fell slowly to an equilibrium value of about 5–10 % of the total.

The ranges of quantities of thyroxine, tri-iodothyronine and compound 3 in the serum of the different rats used in the above-described experiments were: thyroxine, 1·41–6·19 μg./100 ml. of serum; tri-iodothyronine, 0·12–1·90 μg./100 ml. of serum; compound 3, 0·15–0·91 μg./100 ml. of serum.

It is assumed that the total iodine values in the serum remain constant under normal conditions and that the small amount of carrier-free radioactive iodine injected did not disturb this level.

In these experiments the incorporation of ^{131}I into different organic compounds has been compared with the values of ^{127}I in each fraction studied. The results are expressed in terms of specific radioactivity. These data are presented in Fig. 4. The figure shows that in the rats killed half an hour after the administration of radioactive iodide, the specific radioactivity of tri-iodothyronine was much greater than that of the other organic fractions. Its value increased in the first few hours, then diminished rapidly; 6–8 hr. after injection the decrease became slower. The specific radioactivity of thyroxine was very low at the beginning and increased throughout the period studied, finally becoming higher than that of tri-iodothyronine. The specific radioactivity of compound 3, initially higher than that of thyroxine, subsequently decreased to a lower value.

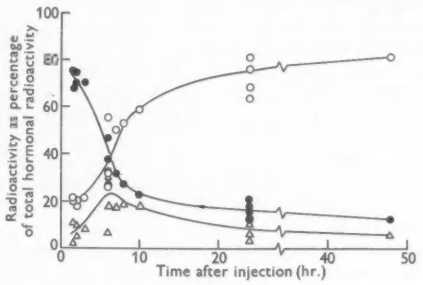

Fig. 3. Formation of thyroid hormones *in vivo* as measured in rat blood after administration of K ^{131}I. For details see the Methods section. ○, Thyroxine; ●, tri-iodothyronine; △, compound 3.

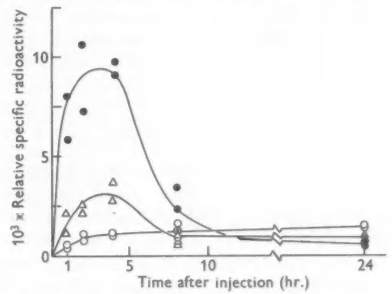

Fig. 4. Relative specific radioactivity of thyroid hormones in rat blood after administration of K ^{131}I. ○, Thyroxine; ●, tri-iodothyronine; △, compound 3.

Table 1. *Effect of thyrotropic hormone on the distribution of radioactive iodine in the thyroid hormones of rat blood*

Adult rats, 200–300 g. body wt., were used throughout. Details of injections and analysis are given in the Methods section.

		^{131}I (as percentage of total hormonal radioactivity)			
		After 6 hr.		After 24 hr.	
Expt. no.	Compound measured	Without thyrotropin	With thyrotropin	Without thyrotropin	With thyrotropin
1	Thyroxine	46·8	65·6	57·6	55·6
	Tri-iodothyronine	49·5	32·0	40·0	42·2
	Compound 3	4·7	2·7	2·4	2·3
2	Thyroxine	38·6	72·0	76·8	85·8
	Tri-iodothyronine	54·2	24·2	13·2	8·5
	Compound 3	7·2	3·8	7·2	5·6
3	Thyroxine	26·2	61·8	55·7	63·0
	Tri-iodothyronine	46·8	24·8	29·7	22·3
	Compound 3	27·0	13·4	15·7	14·8
4	Thyroxine	51·1	81·0	62·3	66·6
	Tri-iodothyronine	31·0	15·5	33·6	27·4
	Compound 3	17·9	3·7	3·9	5·7
5	Thyroxine	57·9	71·2	66·0	63·6
	Tri-iodothyronine	41·0	22·8	23·8	27·5
	Compound 3	4·9	6·7	9·2	8·8
6	Thyroxine	38·7	70·0	70·0	76·2
	Tri-iodothyronine	52·0	27·6	26·7	20·7
	Compound 3	9·3	2·7	3·2	3·1

Effect of thyrotropic hormone on new synthesis of thyroid hormones

In these experiments male albino rats were used. The animals were distributed into different groups, two or three animals constituting a group. One group was given radioactive iodine alone, other groups were given either thyrotropic hormone and radioactive iodine together or the iodine 24 hr. after the injection of the hormone. After suitable periods the radioactivity of the thyroxine, tri-iodothyronine and other iodine compounds in the blood was determined.

The data are presented in Table 1. The percentage radioactivity of thyroxine was higher and that of tri-iodothyronine was lower 6 hr. after the previous injection of thyrotropic hormone, compared with the control values, which suggested that the formation of thyroxine was accelerated by the thyrotropic hormone.

Effect of thyrotropic hormone on the secretion of the thyroid hormones

In further experiments the action of thyrotropin on the quantities of the thyroid hormones secreted was studied in adult rabbits. Fig. 5 represents the change of the protein-bound iodine of rabbit serum at different times after the administration of the thyrotropic hormone. The protein-bound iodine content of the blood increased rapidly after the injection of thyrotropic hormone and reached a steady value in about 24 hr.

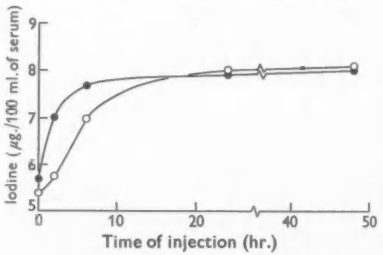

Fig. 5. Change of the iodine content of rabbit serum after the administration of thyrotropic hormone. Curves are the results of two experiments on individual animals.

When the administration of the thyrotropic hormone was repeated between 10 and 20 days after the initial injection, in most cases the opposite phenomenon was observed, the injection of the hormone resulting in a lower protein-bound iodine content of the blood. A third injection 10–20 days after the second once again lowered the levels of protein-bound iodine. Table 2 shows the iodine content of the whole blood and the protein-bound iodine in serum after the first, second and third injection of thyrotropic hormone.

In several experiments the hormones were extracted from the serum, separated and quantitatively determined. The results are summarized in Table 3. Parallel with the change in protein-bound iodine, thyroxine showed the most marked change of all the thyroid hormones. Its quantity in

Table 2. *Effect of thyrotropic hormone on the total and protein-bound iodine of rabbit blood*

Rabbits, 2·5–4·0 kg. body wt., were given thyrotropin as stated. Analysis was done as described in the Methods section.

Expt. no.	No. of injections	Thyrotropin injected (units)	Time of sampling after injection (hr.)	Period between injections (days)	Iodine content (μg./100 ml. of blood)			
					Whole blood		Protein-bound iodine	
					Without thyrotropin	With thyrotropin	Without thyrotropin	With thyrotropin
1	1	50	24	0	15·4	18·2	4·7	9·6
	2	50	6	16	19·1	10·2	4·8	4·2
2	1	25	24	0	7·2	13·7	1·4	4·9
	2	25	24	13	21·0	11·9	6·9	5·5
3	1	25	24	0	15·6	16·9	3·6	4·4
	2	50	24	10	24·9	19·6	4·8	4·4
4	1	50	24	0	16·1	18·2	4·2	6·9
	2	50	24	14	21·6	8·4	9·3	5·8
5	1	50	24	0	2·3	4·9	0·6	1·6
	2	50	24	9	18·5	15·3	7·1	8·3
6	1	50	24	0	5·2	6·8	3·6	4·4
	2	50	24	14	9·3	6·2	4·8	5·2
	3	50	24	15	28·0	17·6	8·5	9·6
7	1	50	24	0	13·2	11·1	3·6	4·4
	2	50	6	10	21·6	8·4	6·8	3·2
	3	50	24	18	16·2	10·5	5·0	2·7
8	1	50	24	0	23·4	28·1	5·7	8·0
	2	50	24	17	28·6	20·3	7·7	4·8
	3	50	24	12	9·0	7·6	8·0	2·7

the serum increased after the first injection of thyrotropin and decreased after the second or third injection. The amount of tri-iodothyronine and compound 3 altered only slightly.

DISCUSSION

The experiments described above have shown that after the intravenous or intraperitoneal injection of radioactive iodine into the rat, the radioactive iodine was rapidly incorporated into the tri-iodothyronine fraction; this compound was formed first and contained the highest proportion of the total radioactivity shortly after the injection. Later on, the proportion of radioactivity increased in the thyroxine fraction (cf. Fig. 3).

Current hypotheses about the mechanism of formation of thyroxine have been summarized (Gross & Pitt-Rivers, 1953; Roche & Michel, 1955) and are two: (*a*) thyroxine is formed by the coupling of two molecules of di-iodotyrosine (cf. Harington, 1944), and thereafter tri-iodothyronine by dehalogenation of thyroxine; (*b*) tri-iodothyronine is first formed by the coupling of one molecule of monoiodotyrosine to one molecule of di-iodotyrosine, thyroxine being formed from tri-iodothyronine by addition of an atom of iodine.

Although Pitt-Rivers (1948) had succeeded in demonstrating the formation of a thyroxine derivative *in vitro* by coupling two di-iodotyrosine derivatives, subsequent work (Gross & Pitt-Rivers, 1953; Roche & Michel, 1955) has not yielded evidence sufficient to establish this as a major route of synthesis of thyroxine. In a re-examination of this type of mechanism Pitt-Rivers & James (1958) have shown that *N*-acetyl-DL-di-iodotyrosyl-1-glutamic acid was converted into the corresponding thyroxine derivative with a 35% yield.

Dehalogenation of thyroxine to tri-iodothyronine has been studied in many tissues. Gross & Leblond (1951) have demonstrated that [^{131}I]thyroxine was dehalogenated *in vivo* to tri-iodothyronine. Albright, Larson & Trist (1954) and Tata (1958) have shown that slices of kidney and homogenates of brain and skeletal muscle can dehalogenate thyroxine. However, such deiodination has not been found to occur in the thyroid gland (Roche, Michel, Lissitzky & Michel, 1951; Roche, Michel, Michel & Lissitzky, 1951), which leads to the suggestion that tri-iodothyronine is formed directly from a mono- and di-iodotyrosine rather than by dehalogenation of thyroxine. Attempts to confirm this suggestion by Gross & Pitt-Rivers (1953) have been unsuccessful.

It is thus seen that the two hypotheses of thyroxine formation have been derived from experiments conducted largely *in vitro*, whereas the present experiments were carried out while hormone formation was proceeding largely undisturbed. Inspection of Figs. 3 and 4 shows that shortly after the administration of radioactive

iodine the proportion and the relative specific radioactivity of tri-iodothyronine is many times greater than that of thyroxine, indicating that synthesis of tri-iodothyronine precedes synthesis of thyroxine. These data seem to be in good agreement with the precursor–product relationship described by Zilversmit, Entenman & Fishler (1943).

In previous experiments measurements of the distribution of radioactivity in the hormones of the thyroid gland over a period of 24 hr. after the injection of ^{131}I yielded results similar to those in Fig. 3 (Feuer & Vekerdy, 1958); this indicated that the pattern of release into the blood is similar to the pattern of formation in the gland. However, similar results could be obtained if thyroxine was not uniformly distributed in the thyroid gland and if the tri-iodothyronine arose from a small but highly labelled pool. Though this has not been directly demonstrated, evidence exists which suggests that incomplete mixing of iodine derivatives may occur under certain circumstances (Triantaphyllidis, 1958*a*, *b*). In these experiments the uptake of radioactive iodine to the gland was inhibited by administration of sodium perchlorate 6 hr. after injection of ^{131}I. Four days later measurement of the levels of radioactive and non-radioactive iodine showed that 55% of ^{131}I and only 27% of ^{127}I had been secreted during this period. From the different speeds of secretion of the isotopes it was concluded that secretion of the iodine derivatives of the gland was not a uniform process.

Table 3. *Distribution of the protein-bound iodine between the thyroid hormones of rabbit serum after the administration of thyrotropic hormone*

Details and methods are as given in Table 2.

Expt. no.	No. of injections	Thyrotropin injected (units)	Time of sampling after injection (hr.)	Period between injections (days)	Compound measured	Iodine content (μg./100 ml. of blood) Without thyrotropin	With thyrotropin
1	1	25	24	0	Thyroxine	1·23	2·06
					Tri-iodothyronine	0·10	0·12
					Compound 3	0·08	0·09
					Sum	1·38	2·27
	2	25	24	13	Thyroxine	1·95	1·08
					Tri-iodothyronine	1·13	1·46
					Compound 3	1·96	1·26
					Sum	5·08	3·80
2	1	25	24	0	Thyroxine	2·00	2·20
					Tri-iodothyronine	1·15	1·46
					Compound 3	0·84	1·10
					Sum	3·99	4·76
	2	50	24	10	Thyroxine	1·98	1·09
					Tri-iodothyronine	1·22	0·84
					Compound 3	1·44	0·96
					Sum	4·54	2·89
3	1	50	24	0	Thyroxine	2·02	2·35
					Tri-iodothyronine	1·38	1·76
					Compound 3	1·10	1·00
					Sum	4·50	5·11
	2	50	24	14	Thyroxine	4·00	1·91
					Tri-iodothyronine	3·58	3·08
					Compound 3	1·44	1·74
					Sum	9·02	6·73
4	1	50	24	0	Thyroxine	2·13	2·30
					Tri-iodothyronine	1·14	1·46
					Compound 3	0·86	0·98
					Sum	4·13	4·74
	2	50	6	10	Thyroxine	3·02	0·68
					Tri-iodothyronine	0·96	0·41
					Compound 3	0·97	0·09
					Sum	4·95	1·18
	3	50	24	18	Thyroxine	2·59	1·45
					Tri-iodothyronine	1·30	0·29
					Compound 3	0·80	0·49
					Sum	4·69	2·23

These experiments are not considered to invalidate the present conclusions. Thus the secretion of the total iodine only was studied during a relatively long period, and not the incorporation of iodine into the individual hormone during shorter intervals. Also, inhibition of sodium perchlorate involves a change in the normal function of the thyroid gland. In the present work the metabolism of the gland was not disturbed in this manner.

The process of formation of thyroid hormones was accelerated by thyrotropin. Thus 6 hr. after the injection of thyrotropic hormone the radioactivity of thyroxine was higher and that of tri-iodothyronine was lower than the values in control animals (Table 1). It is considered that the results show that thyrotropic hormone has an effect not only on the secretion of the thyroid hormones but also on the transformation of tri-iodothyronine into thyroxine.

Repeated injections of thyrotropin into rabbits at intervals of 10–20 days decreased the levels of protein-bound iodine after each injection subsequent to the first, which produced the well-known increase (see Table 2). Reasons for this phenomenon are not known. In other animals, such as the guinea pig, repeated daily injections of thyrotropin consistently produce the expected response (cf. Adams & Purves, 1955). It is possible that the continuous high level of blood thyroxine persisting after the initial dose renders the thyroid in the rabbit less responsive to further endogenous thyrotropin, or that thyrotropic hormone exerts a peripheral effect on thyroid-hormone metabolism in addition to its action on the thyroid gland, but in the absence of further data such suggestions must remain hypothetical.

SUMMARY

1. The formation of thyroid hormones *in vivo* has been studied in the blood of different animals with the aid of a new micromethod.

2. After an injection of radioactive iodine into rats, it was found that the radioactivity of tri-iodothyronine initially exceeded that of thyroxine, later becoming much less. It is thought that tri-iodothyronine is a precursor of thyroxine.

3. Six hours after the injection of thyrotropic hormone, together with radioactive iodine, the radioactivity of the blood thyroxine was much higher, and that of tri-iodothyronine much lower, than that of the controls. Thyrotropic hormone has an accelerating effect on the transformation of tri-iodothyronine into thyroxine.

4. After an injection of thyrotropic hormone into rabbits, the protein-bound iodine of the blood increased. Second or third injections at intervals of 10–18 days produced a decrease.

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The Purification of the Proteolytic Component of Elastase

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Of the many hypotheses advanced to explain the onset of atherosclerosis, one (Baló & Banga, 1949) implicates the pancreatic enzyme elastase and its inhibitor (Baló & Banga, 1949; Graham, 1958). Since these first claims, interest in elastase has not diminished, although the simplicity of the original concept has been considerably impaired by the realization that not only is the level of the circulating inhibitor lowered in old age, but the elastase content of the pancreas is also decreased (Baló & Banga, 1953). Many studies of the interaction between the protein elastin, or the intact elastic fibre and the enzyme have been reported (Balo & Banga, 1949; Lansing, Rosenthal, Alex & Dempsey, 1952; Hall, Reed & Tunbridge, 1952, 1955).

A number of methods have been described for the preparation of elastase (Baló & Banga, 1950; Banga, 1952; Bagdy & Banga, 1958; Hall & Gardiner, 1955; Hall, 1957; Grant & Robbins, 1957; Lewis, Williams & Brink, 1956). Most of these appear to have certain failings. In all the earlier papers it was realized that the enzyme was not necessarily pure and Bagdy & Banga (1958) record that their preparation is not electrophoretically pure. Lewis *et al.* (1956) claimed electrophoretic homogeneity for a preparation obtained from their 'crystalline' material, but in the authors' Laboratories this also had a polydisperse appearance after paper electrophoresis.

Hall (1953, 1957) showed that at least two active fractions, E_2 and E_1, were present in crude preparations, and it became clear that two steps were necessary in any preparative procedures, namely: the separation of the active material from the other components of crude pancreas extract and the division of the active material into its two separate entities. Electrophoretic separation proved adequate for both these steps but the yields so obtained were insufficient for kinetic studies on E_2 (Czerkawski, 1958) or studies on E_1 *in vivo* (Saxl, 1957, and unpublished work, 1959); Hall, 1958 and unpublished work, 1959). The present paper reports a method for the bulk preparation of the fraction E_2 in an electrophoretically pure state.

EXPERIMENTAL

Materials

Most of the reagents used were of A.R. grade but a good technical grade of aluminium sulphate proved adequate for the preparation of the gel, provided that the number of molecules of water of crystallization was known. Dialyses were carried out in Visking cellophan tubing (2 in. diam.) against approx. 3 vol. of resin-demineralized water at 5°.

Pancreas powder. As in previous studies from these Laboratories (Hall, 1955; Hall & Gardiner, 1955; Hall, 1957), a commercial hog-pancreas powder was employed as starting material in preference to fresh pancreas, since this enabled more constant recoveries of activity to be achieved. The source of the powder (Viokase) was Viobin Inc., Monticello, Ill., U.S.A.

Elastin. Ox ligamentum nuchae powder passing a 40 but retained by a 60 mesh/in. sieve was employed throughout. Ground substance and collagen were removed as reported previously (Hall, 1955) by autoclaving with 2% (v/v) acetic acid.

Alumina gel. Aluminium hydroxide in the form of the C-γ gel was prepared by a modification of the method of Willstätter & Kraut (1923). The gel was formed by the addition of the following two reagents: *A*, 90 g. of $(NH_4)_2SO_4$ was dissolved in water, 83·1 ml. of aq. NH_3 soln. (sp.gr. 0·88) was added and the whole diluted to 1950 ml.; *B*, 142·7 g. of $Al_2(SO_4)_3.16H_2O$ was dissolved in a little water and the solution diluted to 300 ml. Reagent *A* was maintained at 60° and *B* was added slowly with stirring. After further stirring at 65° for 15 min. the suspension was poured into 18 l. of tap water (soft, calcium and magnesium salts 50 p.p.m.), at room temperature, containing 50 ml. of aq. 2N-NH_3 soln. The gel was allowed to settle overnight and after the supernatant had been removed by decantation, was washed twice with aq. 0·05N-NH_3 soln. Great care was taken to ensure that the gel never became acid, during either its preparation or its subsequent use.

Measurement of protein concentrations in enzyme preparations

Of the numerous methods available, u.v.-absorption at 280 mμ was standardized as the most suitable method, being least dependent on the composition of the protein but having a sensitivity suitable for the measurement of microgram quantities of enzyme.

Elastolysis. The extent of elastolysis in terms of the material passing into solution was measured by means of an interferometer (Claesson, 1946) and expressed as percentage of protein dissolved, on the original air-dried weight of substrate (moisture content of air-dried material 14·7%).

The vessel containing the reaction mixture replaces the adsorption column in the apparatus as originally designed by Claesson (1946). It is thus mounted on the top of the cuvette which lies in one of the light paths of the Rayleigh interferometer, but is separated from it by a filter disk. At frequent intervals throughout the course of the reaction, small samples can be drawn into the cuvette by suction, the increase in refractive index can be measured and then

the samples are returned to the reaction vessel. The refractive-index increments of soluble elastin, NaCl and a soluble polysaccharide do not vary by more than 5%, and since the non-protein content of elastin is less than 5% the error in using a value of 0·018 for the increment is less than 0·5%. Hence the method estimates the total amount of solid passing into solution and replaces laborious gravimetric estimations.

Definition of an elastase unit. The elastase unit (e.u.) is defined as that amount of elastase which dissolves 1 mg. of ox ligament (initial concentration, 5 mg. of solid/ml.) in 3 hr. at 37°, the buffer used being borate, pH 8·7, I 0·364. The amount of enzyme is chosen so that not more than 60% and not less than 20% of the initial substrate is hydrolysed, since under these conditions the activity is on the essentially linear central portion of a sigmoid curve. The definition differs considerably from those of other workers (see Discussion).

Electrophoresis. The progress of the purification was followed throughout by high-voltage paper electrophoresis. The apparatus was of the 'sandwich' type, in which the paper (Whatman no. 3 MM) was clamped tightly between the upper and lower surfaces of two Perspex cooling chambers. In every case standard conditions of 16 v/cm., giving a 60 mA current across a paper (55 cm. × 12 cm.) in veronal buffer, pH 8·6, I 0·15, were used. Protein constituents were dyed, after fixing by heating at 140° for 60 min., by immersion for 10 min. in a hot (65°) 0·05% solution of Solvay Purple in aq. 0·5% H_2SO_4. Surplus dye was rinsed off with warm water.

Method of purification

Pancreas powder (200 g.) was shaken gently for 15 min. with 1 l. of acetate buffer, pH 4·7, I 0·1, and allowed to stand at 5° for 5 hr. This was then centrifuged at 5° at 1890 *g* and the residue extracted for a further 16 hr. with 1 l. of the same buffer, under identical conditions. The supernatants were pooled and the residues discarded. The supernatant, which was a suspension of very fine particles, was centrifuged again at high speed (22 700 *g*) at 5° when a clear golden-yellow liquid was obtained.

First dialysis. The acid extract was dialysed at 5° against 3 l. of water for 3 days, the water being changed at least twice a day. After this time interval, a copious precipitate of euglobulin was formed, and this was removed by centrifuging at 5° at 1890 *g* for 15 min., followed by decantation. The euglobulin was kept at 5° while the supernatant was subjected to further dialysis under the same conditions as those described above. This was repeated daily until precipitation was complete, usually after 2 days of further dialysis. The final supernatant and the precipitates could be freeze-dried at this stage to give preparations AES and AEI (Fig. 1), their differences in properties (Table 1) indicating that the majority of the proteolytic enzyme was segregated in the euglobulin fraction (AEI). The euglobulins were pooled and suspended in 200 ml. of cold water, the suspension was centrifuged and the supernatant decanted. The wet solid was then resuspended in 500 ml. of water and finely powdered NH_4HCO_3 added with stirring until the whole protein was dissolved. At this stage the solution could be freeze-dried and kept indefinitely at 5°

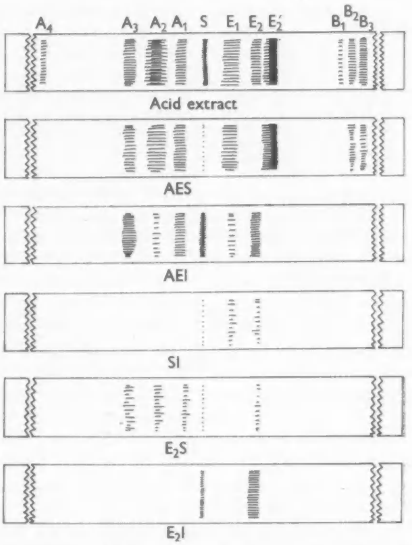

Fig. 1. Electrophoresis diagrams of fractions of elastase obtained at various stages during the purification of the proteolytic component E_2. Electrophoresis was carried out as described in the text. S, Stationary material adsorbed at point of application; E_1, so-called 'mucolytic fraction' (Hall, 1957); E_2, proteolytic fraction; E_2', fraction preventing synergistic effects apparent when E_1 and E_2 are tested together; A_1–A_4, acidic fractions having no effect on elastolysis; B_1–B_3, basic fractions having no effect on elastolysis.

Table 1. *Elastolytic activity of fractions*

All the values listed were calculated on the dry weight of each sample. Values of the fifth column were determined by the method described in the text.

Fraction	Water content (%)	Nitrogen content (%)	Yield (g.)	Specific activity (e.u./mg.)	10^3 × Total no. of e.u.
Pancreatic powder	8·9	10·7	182·0	8·5	1560
AES	3·7	11·1	27·0	6·2	168
AEI	13·0	14·7	7·1	86·5	612
SI	12·3	7·5	0·91	22·8	22
E_2S	11·0	9·4	1·51	16·3	25
E_2I	14·5	14·5	0·40	368·0	145

until required for further purification, or alternatively the solution could be used as such for the next step of the purification procedure.

Adsorption. The solution of euglobulin was made slightly alkaline (pH 7·5–8·0) with aq. 2N-NH_3 soln. and mixed with the C-γ gel. The total volume was brought to 2 l. with water and the suspension shaken gently for 15 min., after which it was allowed to stand at 5° overnight. The suspension was centrifuged and decanted, and the residue of C-γ gel, together with adsorbed elastase, was washed with 200 ml. of cold water. The supernatant was dialysed against tap water for 3 days and freeze-dried to give a fraction S1.

Extraction and second dialysis. The gel was shaken gently with 1·25 l. of 0·2M-Na_2HPO_4 solution, containing enough HCl to give pH 8·0–8·5, and allowed to stand at 5° for 45 hr. with intermittent stirring. The suspension was then centrifuged and the residue washed with 0·75 l. of the same acidified phosphate solution. The supernatant and washings were dialysed under the same conditions as those used in the preparation of the euglobulin fraction from the original acid extract. A precipitate appeared after 2 days, and precipitation was complete after 5 days. The supernatant was freeze-dried to give fraction E_2S and the solids were pooled and washed with a little cold water. The solid was dissolved in 1 l. of M-NH_4HCO_3 soln. and filtered before freeze-drying to give fraction E_2I.

RESULTS

Electrophoretic examination of intermediate stages in the purification. As can be seen from Fig. 1 the crude acid extract contains at least nine bands: three acid components, a stationary band S, the important components E_1 and E_2 and three strongly basic components B_1, B_2 and B_3. There is also a band E_2', which has practically the same mobility as E_2 and which stains to a grey shade with Solvay Purple as compared with the E_2 which stains purple with the dye. This compound is only separable from E_2 in buffers of relatively high ionic strength such as are employed here. The material in band E_1 appears to be very soluble and becomes visible only after prolonged heating before staining. Components of this band appear to have a synergistic effect on the activity of E_2, which is the only electrophoretic component showing elastolytic activity when examined alone.

The first dialysis gives the fractions AES and AEI. The former retains all A_2 and little A_1, most of the basic components B and nearly all E_1 and E_2'. The fraction AEI is still rich in the elastolytically inactive component A_3; it contains a large amount of E_2 and a relatively large amount of insoluble material. The fraction S1 contains a little E_1 and E_2', and very little of the unadsorbed E_2 component. The second dialysis confines the small amount of acidic material to fraction E_2S to give a fraction E_2I, which consists solely of E_2 with a small amount of insoluble material. By centrifuging this can be removed from E_2 solutions in buffer or sodium chloride without any loss of activity.

Isoelectric point. Several electrophoresis runs were made in veronal buffers, I 0·15, having the pH indicated in Table 2. Otherwise, standard conditions were used and, to correct for electroendosmosis, glucose was subjected to electrophoresis simultaneously. It will be seen from the figures in Table 2 that after making the necessary corrections the isoelectric point appears to be near pH 8·5.

Effect of pH *on activity.* Hall & Gardiner (1955) obtained curves relating activity to pH with a relatively impure enzyme, and showed that for this material the curve has two maxima, at pH 7·8 and 8·7, the upper of which was ascribed to E_2 (Hall, 1957). With the purified enzyme E_2I, only one maximum, at pH 8·45, is obtained.

Specific activity. It will be seen from Table 1 that, within the limits of experimental error, the purification produced after the first two steps is nearly tenfold, whereas the complete procedure gives a material which is about 40 times as active as the starting material; the question of the validity of these figures is considered in the Discussion section.

Variation of extent of elastolysis with substrate concentration. Table 3 shows a typical set of results illustrating the variation of extent of elastolysis with the initial amount of substrate at different time intervals. It will be seen that the percentages dissolved in corresponding time intervals approach a constant value when the substrate content

Table 2. *Determination of the isoelectric point of elastase by paper electrophoresis*

Conditions used were 60 mA, 16 v/cm., 1¾ hr. with veronal buffers, I 0·15.

pH	Distance moved by E_2 (cm.)	Distance moved by glucose (cm.)	Net distance moved by E_2 (cm.)
7·6	+3·5	+2·6	+0·9
8·6	+2·2	+2·3	−0·1
9·6	+1·4	+2·5	−1·1
11·0	+0·5	+2·5	−2·0

Table 3. *Variation of elastolytic activity with substrate/enzyme ratio*

Enzyme concentration was constant and equal to 54 μg./ml.

Starting amount of substrate (mg./10 ml.)	Percentage of elastin dissolved		
	Time 1 hr.	Time 2 hr.	Time 3 hr.
10	36	84	100
20	28	70	94
30	24	62	88
40	22	57	83
60	20	56	80

reaches about 6 mg./ml. It was also found that the curve relating activity to enzyme concentration is linear only for relatively low enzyme concentration (not higher than 4·0 μg. of E_2I/ml.) (Figs. 2, 3.)

Ultraviolet absorption of E_2I. Absorption spectrum of E_2I dissolved in 0·1 % sodium chloride and in Clark and Lubs 0·5 M-borate buffer, pH 8·7, was determined. In each there was a definite maximum at a wavelength of 275 mμ. Extinction was proportional to concentrations up to 500 μg./ml., giving an extinction coefficient of 13.

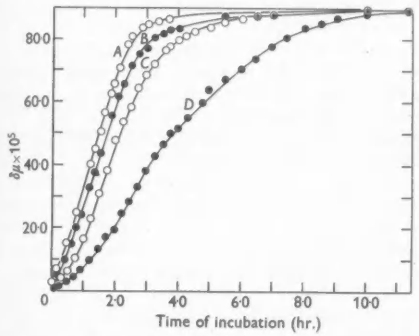

Fig. 2. Activity (expressed in terms of increased refractive index of supernatant)–time curves for various concentrations of E_2: *A*, 11·4; *B*, 7·6; *C*, 3·8; *D*, 1·9 μg./ml. In each, 5 mg. of solid elastin/ml. was used.

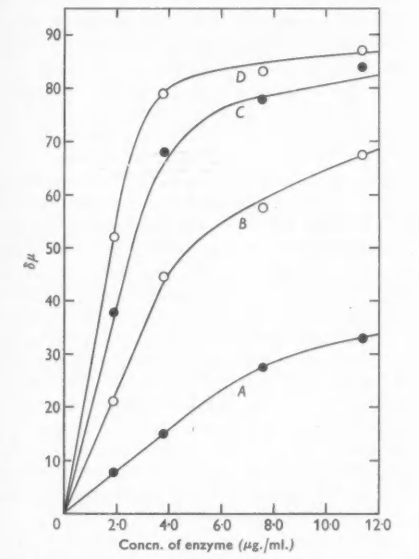

Fig. 3. Relationship between activity (expressed as increases in refractive index of the supernatant) and concentration of enzyme, for four different periods of incubation: *A*, 1; *B*, 2; *C*, 3; *D*, 4 hr. In each, incubation was carried out at 37° with an initial substrate concentration of 5 mg./ml.

DISCUSSION

The progress of the elastolytic reaction, measurement of which is an essential for the assessment of any purification of elastase, has been estimated by a variety of methods. These include qualitative methods based on histological sections and electron-microscope studies (Baló & Banga, 1949; Saxl, 1958), gravimetric methods (Baló & Banga, 1950), biuret estimation of the protein released into solution (Hall & Gardiner, 1955; Hall, 1955) and the measurement of dye released into solution after the action of the enzyme on a previously dyed elastin preparation (Sachar, Winter, Sicher & Frankel, 1955; Roberts & Samuel, 1957).

Of these the histological method evaluates the enzyme activity under conditions as near physiological as possible, but the results can be no more than semiquantitative. The determination of protein release measures only one aspect of the whole effect of the enzyme whereas the solubilization of the dyed tissue may represent a reaction far removed from the normal physiological conditions. In a molecule with so few polar groups as elastin, sites of dye attachment may well be intimately related to those at which the enzyme becomes attached. Findlay (1954) states that staining with resorcinol fuchsin inhibits elastase attack on whole tissue but not on powdered material, and with the latter tissue which has been pre-stained it is conceivable that the points of attack are quite dissimilar to those in fresh tissue.

The gravimetric method measures solubilization of all the components of the tissue, and the interferometric method described here permits this effect to be measured continuously throughout a reaction without the tedious manipulations of the gravimetric method.

One of the difficulties which has arisen over the past few years about the comparison of the results for elastase activity provided by a variety of workers has been the differences in the criteria on which they have based these measurements. Four or five definitions of a unit for elastase have been proposed. Baló & Banga (1950) first defined an elastase unit as that amount of elastase which dissolves 1 mg. of elastin in 0·5 hr. This unit has been used widely but, as will be seen from the following discussion, it is very artificial and rather unsatisfactory. Such a unit should be examined critically in the light of the latest developments reported here and by other workers. The

elastolysis–time curve is sigmoid, which makes it difficult to identify the percentage elastolysis after a given time as being identical with the classically defined 'initial reaction velocity'. There is experimental evidence that the solubilizing action of elastase is accompanied by a certain amount of purely hydrolytic action, in which the large soluble protein fragments are split into smaller polypeptides (Partridge & Davis, 1955). This implies that the function of the E_2 component is dual in nature and that at any one time only a part of the available enzyme is concerned in solubilization, which is the measurable effect. It follows therefore that the magnitude of the elastase unit will vary with the substrate/enzyme ratio (cf. Table 3). It can be shown that the number of elastase units in a given preparation increases considerably with the starting amount of elastin, provided that enzyme concentration remains constant (Fig. 4).

Many workers have used buffer solutions of relatively low ionic strength. This enables a higher specific activity to be recorded for the enzyme, since activity varies inversely with salt concentration (Lewis *et al.* 1956) and consequently the results of purification procedures can appear very attractive. It was decided that rather than sacrifice reproducibility, it would be better to use strongly buffering solutions such as borate (I 0·36), with its unavoidable decrease in activity, especially in a system such as this which is accompanied by the liberation of free acid.

Further, the time interval of 0·5 hr. is too short because to get a measurable effect with relatively inactive fractions of crude tissue extracts it is necessary to use large amounts of protein material. Lewis *et al.* (1956) in their paper on the preparation of elastase report that the incubation period had to be extended to 2 hr. when certain preparations were estimated, thus invalidating direct comparisons such as they make between various stages in the purification.

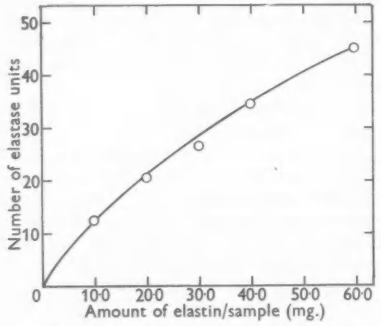

Fig. 4. Relationship between the activity of a given elastase fraction (here E_2) and the amount of substrate initially available for reaction. Each point represents results obtained for a system containing 5·4 μg. of E_2/ml. and incubated for 3 hr. at 37°.

A further complication in the definition of an elastase unit is the observation of Hall (1957) that the component E_1, although elastolytically inactive, increases the activity of E_2 by nearly 50%. This means that the specific activity of many of the early fractions is higher than would be expected for a given amount of E_2 and in the extreme case a fraction consisting mostly of E_1 with little E_2 would show a specific activity, completely out of keeping with its E_2 content.

Although the primary object of this method of preparation is to obtain the elastolytic component E_2, at the same time it throws some light on the possible means of isolation of component E_1, which apart from its synergistic action on E_2 has been shown (Saxl, 1957, 1958) to be of importance in the solubilization of the mucopolysaccharide portion of elastic fibres.

It can be seen from the investigation of the enzyme–substrate reaction that the best conditions under which to define an elastase unit would be such that enzyme concentration is relatively small and the initial amount of substrate is as large as possible. The ensuing difficulties of measurement under such conditions and the presence of a large excess of reaction products would necessitate limiting the reaction to about one-fifth of its complete course. There is every indication from the shape of the curve (Fig. 2) that the reaction is not of the same type from beginning to end, so that the first fifth of its course may not be a true representation of the elastolytic reaction as a whole, and it is safer until such time as the processes concerned are fully characterized to make measurements near the region of 50% elastolysis.

It can be seen from the foregoing that it is very difficult to evolve a really satisfactory elastase unit. Work is now in progress and preliminary reports have been made (Czerkawski, 1958), which it is hoped will lead to the elucidation of the kinetics of the whole elastin–elastase system; this would then provide a sound basis for definition of the elastase unit. However, until such a time it is thought that the unit defined in the Experimental section utilizes the best possible conditions, is convenient and is capable of reproduction.

It must be stressed that for good reproducibility one must adhere to the stated conditions, especially for initial amount of substrate. Fig. 3 shows the increase in the number of elastase units contained in a given sample of elastase with increasing initial substrate concentration. Since the actual amount of enzyme used is constant the real value of the

elastase unit becomes smaller with increasing elastin concentration.

An important point emerges from the successful purification of the proteolytic component of elastase. Partridge & Davis (1955) have shown that soluble elastin prepared by the oxalic acid extraction of solid elastin can be fractionated into two proteins, only one species, the α-elastin, producing coacervation on warming at low pH. This property of coacervation was rapidly destroyed by a crude elastase preparation such as was used by these authors.

We have found that great amounts of this material can be produced during the middle third of the reaction between solid elastin and purified elastase. When α-elastin obtained in this fashion is subjected to the same enzyme the power of coacervation disappears fairly rapidly, but a crude elastase having much lower elastolytic activity removes the power to coacervate very rapidly indeed.

One could infer from Partridge's work that during enzymic solubilization of elastin, only β-elastin is produced and production of α-material is only inherent in oxalic acid extraction. This would appear not to be so, and the results demonstrate the danger of employing impure enzymes until adequate proof is available that the contaminating proteins play no part in the main reaction. In this context it is of interest that Saxl (1958) has observed the formation of globular degradation products of elastase attack only after the use of highly purified enzyme.

SUMMARY

1. A practical unit of elastase is defined.

2. A method of purification, based on acid extraction, fractional dialysis, adsorption and extraction, is described.

3. Electrophoretic examination of each fraction is reported, together with an estimation of the isoelectric point of the final fraction E_2I. The isoelectric point of elastase appears to be at pH 8·5, which virtually coincides with the single peak of the pH–activity curve.

4. The mode of variation of specific activity of elastase with initial amount of substrate and enzyme is reported. Most suitable conditions for the definition of elastase unit are discussed. Difficulties due to the complex nature of the elastase system are indicated.

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Turnover of Nuclear and Cytoplasmic Ribonucleic Acid in Two Types of Animal Cell, with some Further Observations on the Nucleolus

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In a previous paper (Watts & Harris, 1959) some of the principles underlying the measurement of nucleic acid turnover in animal cells were discussed. It was shown that although in the macrophage a rapid turnover of ribonucleic acid occurs, as measured by the incorporation and release of [8-^{14}C]adenine, the products of turnover are completely re-utilized by the cell in the resynthesis of ribonucleic acid and are thus continuously recycled. Under such conditions the turnover is not detected unless the cells are cultivated in a medium containing some unlabelled compound which has access to the turnover pathway and dilutes or displaces some labelled intermediate in the process of recycling. Of the compounds tested, adenosine was found to be the most effective in this respect.

Measurements of ribonucleic acid turnover in different parts of the cell have been undertaken. These measurements were made possible by the use of tritium-labelled precursors and quantitative radioautographic methods. Two cell types were studied: the connective-tissue cell derived from rat-heart tissue, and the macrophage obtained from the peritoneal cavity of the rabbit. The former multiplies *in vitro*; the latter does not multiply but remains alive for long periods.

In a previous paper (Harris, 1959) some observations were made on the function of the nucleoli, which are prominent structures in the connective-tissue cell. These observations are further elaborated in the present paper.

EXPERIMENTAL

Preparation of cultures

The connective-tissue cells were obtained from the hearts of 5-day-old rats and cultivated as dissociated cells in small coverslip chambers (Harris, 1955). The macrophages were obtained from the peritoneal cavity of the rabbit (Lucké, Strumia, Mudd, McCutcheon & Mudd, 1933) and were maintained as a single-cell layer in similar chambers. The culture medium consisted of 80% (v/v) of Eagle's solution (Eagle, Oyama, Levy, Horton & Fleischman, 1956) with 20% (v/v) of bovine serum for the connective-tissue cells and 20% (v/v) of rabbit serum for the macrophages. The tritium-labelled precursors, adenosine, cytidine and thymidine, were used at a concentration of 15 μc/ml. of medium. These compounds were obtained from Schwarz Laboratories Inc., Mt. Vernon, N.Y., U.S.A., and had specific activities of 1·0c, 0·36c and 1·9c/m-mole respectively; they were all stably labelled in the purine or pyrimidine ring.

Method used for measuring ribonucleic acid turnover

In previous experiments (Watts & Harris, 1959) it was shown that in the macrophage there is no synthesis of deoxyribonucleic acid (DNA). It was also shown that exogenous [^{14}C]adenine contributes no radioactive carbon to the proteins of the cell. The incorporation of adenosine into compounds insoluble in 0·3 N-trichloroacetic acid at 0° can therefore be regarded as incorporation into ribonucleic acid (RNA) in this cell.

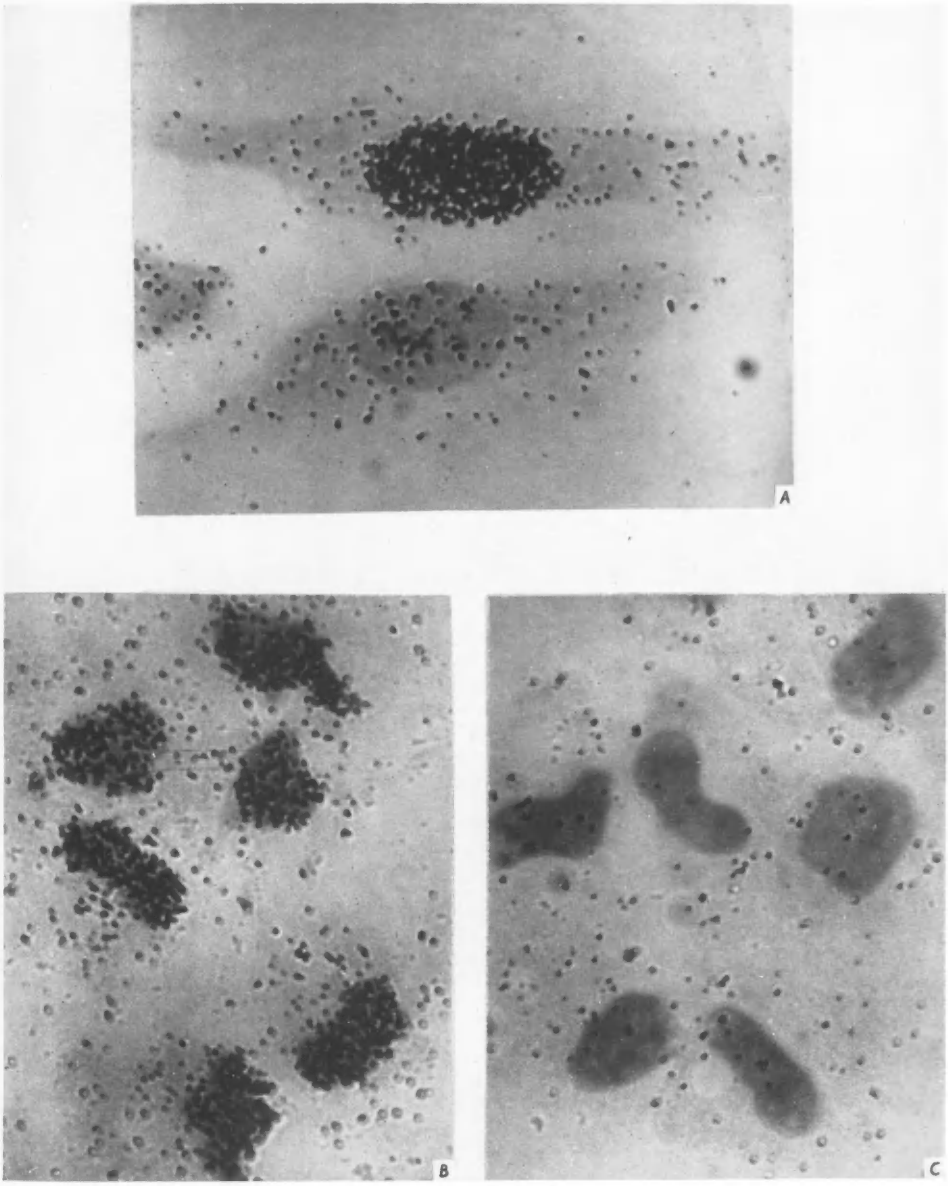
In the multiplying connective-tissue cell, however, synthesis of DNA occurs, and adenosine may be incorporated into both DNA and RNA. But DNA is synthesized only during a certain phase of the intermitotic cycle, and it is only in this phase that precursors are incorporated into DNA (Harris, 1959). Cells which are not in the phase of DNA synthesis incorporate adenosine only into RNA. Thus in order to measure turnover of RNA by the incorporation and release of labelled adenosine it is necessary to select cells which are not in the phase of DNA synthesis. This was achieved in the present experiments by adding tritium-labelled thymidine to the medium as well as tritium-labelled adenosine. Cells in the phase of DNA synthesis

EXPLANATION OF PLATE 1

A. Radioautograph of two connective-tissue cells from the same culture. The culture was exposed to tritium-labelled adenosine and tritium-labelled thymidine for 10 min., washed with three changes of non-radioactive medium containing an excess of unlabelled adenosine and thymidine and then incubated for 3 hr. in non-radioactive medium containing 2 mM-adenosine and 2 mM-guanosine. The cells were fixed at the end of this period. The upper cell was in the phase of DNA synthesis at the time of exposure to the tritium-labelled precursors; the lower cell was not.

B. Radioautograph of macrophages fixed after 30 min. exposure to tritium-labelled adenosine. The heavy labelling is over the nuclei; the lighter labelling is over the cytoplasm, which is not counterstained.

C. Radioautograph of macrophages fixed after incubation for 30 min. in medium containing tritium-labelled adenosine and 8 hr. in non-radioactive medium containing 2 mM-adenosine and 2 mM-guanosine. The nuclei are much less heavily labelled and there are also fewer grains over the cytoplasm.



H. HARRIS—Turnover of nuclear and cytoplasmic ribonucleic acid in two types of animal cell, with some further observations on the nucleolus

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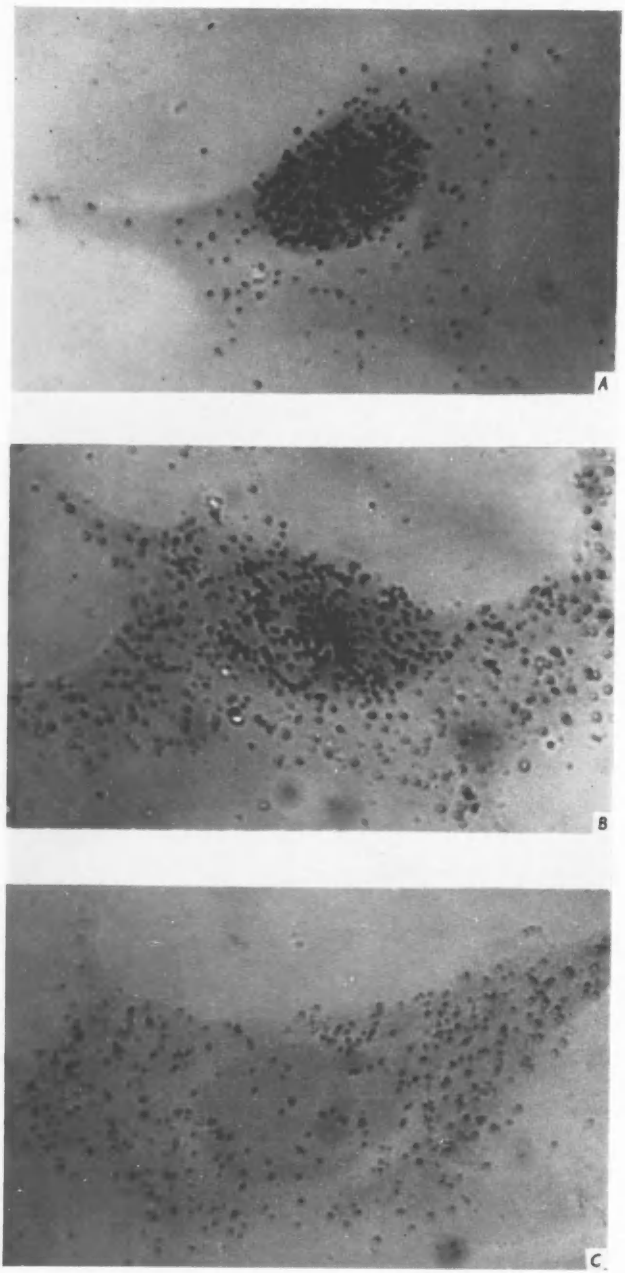

H. HARRIS—Turnover of nuclear and cytoplasmic ribonucleic acid in two types of animal cell, with some further observations on the nucleolus

incorporated both the adenosine and the thymidine into DNA and the adenosine into nuclear RNA. The nuclei of such cells were therefore much more heavily labelled than the nuclei of cells not in the phase of DNA synthesis, since the latter incorporated the adenosine only into RNA and did not incorporate the thymidine at all. The two groups of cells were easily distinguished, as shown in Plate 1 *A*.

Pre-warmed medium containing tritium-labelled adenosine and, with connective-tissue cells, tritium-labelled thymidine, was introduced into ten chambers in which the cells were being cultivated. The cells were incubated at 37° in the radioactive medium for periods of 5–30 min. The radioactive medium was then withdrawn and the cells adherent to the floor of the chamber were washed with three changes of medium containing an excess of unlabelled adenosine and thymidine. Incubation was then continued for periods up to 12 hr. in non-radioactive medium containing 2 mM-adenosine and 2 mM-guanosine. The guanosine was added because some of the radioactivity from exogenous [^{14}C]adenine had been found to appear in RNA guanine residues (Watts & Harris, 1959). Similar experiments were carried out with tritium-labelled cytidine as the precursor; in these 2 mM-cytidine and 2 mM-uridine were added to the non-radioactive medium.

At the end of the period of incubation in the radioactive medium and at regular intervals afterwards the coverslip with the adherent cells was removed from one of the chambers. The cells were washed in 0·85% NaCl soln. and then fixed. The connective-tissue cells, which become extremely flat when grown on a glass surface, were fixed by immersion in methanol for 15 min. The macrophages were flattened before fixation. This was done by pressing the cells between the coverslip and a small square of agar and then allowing the methanol to diffuse across the interspace containing the cells (Slider & Downey, 1950).

After fixation the cells were extracted with 0·3 N-trichloroacetic acid at 0°, washed under running water and then dried. The coverslips, with the cell-covered surface facing upwards, were mounted on slides. Radioautographs were prepared by the stripping-film technique with Kodak AR. 10 film (Pelc, 1956). The films were developed after 4–8 days and the cells stained through the emulsion with nuclear-fast red.

Measurement of the amount of radioactive precursor in various parts of the cell

The amount of radioactive precursor incorporated into any part of the cell is reflected by the number of silver grains visible in the emulsion overlying that part of the cell. The high resolution of radioautographs produced by tritium-labelled compounds is well known. With cells cultivated on glass and fixed in the manner described it was possible to resolve not only nuclear and cytoplasmic radiation, but also radiation emanating from the nucleoli. Owing to the extreme thinness of the fixed dried cells the results were not complicated by variation in the internal absorption of the tritium radiation. Below a certain grain density the relation between the number of visible grains in the emulsion overlying a given part of the cell and the amount of tritium-labelled precursor incorporated in that part of the cell was found to be linear. Fig. 1 illustrates the relation between the number of visible grains over the

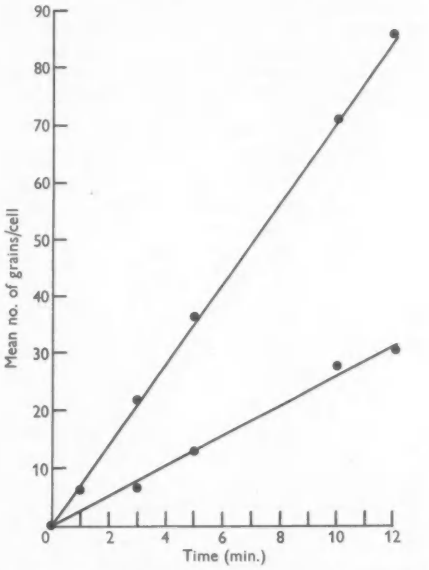

Fig. 1. Relation between the number of visible grains in the emulsion overlying the nucleus and the nucleoli of connective-tissue cells and the time for which the cells were exposed to a tritium-labelled precursor. Cells were incubated in medium containing tritium-labelled adenosine and tritium-labelled thymidine for the periods shown and then fixed. Cells not in the phase of DNA synthesis were selected for grain counts. The lower line shows the mean number of grains per cell over the nucleoli; the upper line shows the mean number of grains per cell over the rest of the nucleus.

EXPLANATION OF PLATE 2

A. Radioautograph of a connective-tissue cell fixed after incubation for 20 min. in medium containing tritium-labelled adenosine. The cell was not in the phase of DNA synthesis. The nucleus is more heavily labelled than the cytoplasm and the heaviest labelling is over the nucleolus.

B. Radioautograph of a connective-tissue cell fixed after incubation for 20 min. in medium containing tritium-labelled adenosine and 3 hr. in non-radioactive medium containing 2 mM-adenosine and 2 mM-guanosine. The cell was not in the phase of DNA synthesis at the time of exposure to the tritium-labelled precursor. The nucleus is less heavily labelled than the nucleus in *A*, but the cytoplasm is much more heavily labelled. The nucleolus is still the most heavily labelled part of the cell.

C. Radioautograph of a connective-tissue cell fixed after incubation for 20 min. in medium containing tritium-labelled adenosine and 12 hr. in non-radioactive medium containing 2 mM-adenosine and 2 mM-guanosine. The nucleus is now much less heavily labelled than in *A* and *B*, but the cytoplasm shows about the same degree of labelling as in *B*.

nucleus and the nucleoli of connective-tissue cells and the time for which the cells were incubated in the presence of tritium-labelled adenosine. The incorporation of the precursor into RNA had previously been shown to be virtually linear over periods of this duration. The length of exposure of the radioautographs was regulated to give grain densities within this linear range.

In each preparation forty macrophages or forty connective-tissue cells not in the phase of DNA synthesis were chosen at random for grain counts. This was found to be a large enough sample: the standard error of the mean number of grains over the nucleus, nucleolus or cytoplasm was always less than 10% of the mean.

RESULTS

Macrophages

Since it has been shown that the amount of RNA per cell remains constant (Watts & Harris, 1959), the release of labelled adenosine from the RNA is a measure of RNA turnover. The turnover of nuclear and cytoplasmic RNA in the presence of exogenous unlabelled adenosine and guanosine is shown in Fig. 2. The observed half-life of the loss of radioactivity from the nucleus is less than 2 hr.; the observed half-life of the loss from the cytoplasm is between 5 and 7 hr. Plate 1*B* shows a radioautograph of cells fixed after incubation for 30 min. in the radioactive medium. Plate 1*C* shows a radioautograph of cells from the same experiment which were incubated for 30 min. in the radioactive medium and then for 8 hr. in the non-radioactive medium before fixation. It will be seen that there has been a much greater fall in the number of grains over the nucleus than in the number over the cytoplasm. For purposes of illustration these radioautographs were exposed for a longer period than was optimum for grain counting.

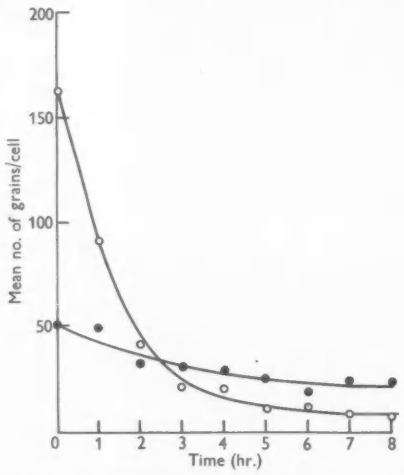

Fig. 2. Turnover of nuclear and cytoplasmic RNA in the macrophage. ○, Nuclear RNA; ●, cytoplasmic RNA. Cells were exposed to tritium-labelled adenosine for 30 min., washed with three changes of non-radioactive medium containing an excess of unlabelled adenosine and then incubated for 8 hr. in non-radioactive medium containing 2 mM-adenosine and 2 mM-guanosine. The incubation in non-radioactive medium was begun at zero time.

The curves shown in Fig. 2 appear to set very low limits to the amount of nuclear RNA which could have passed into the cytoplasm, unless the nuclear RNA were rapidly destroyed on entering the cytoplasm. If as little as 10% of the labelled RNA in the nucleus had passed unchanged into the cytoplasm in an hour, it should have been detected by the techniques used.

Connective-tissue cells

Fig. 3 shows the turnover of the nuclear and cytoplasmic RNA of the connective-tissue cell in the presence of exogenous unlabelled adenosine and guanosine. In this cell the number of grains over the cytoplasm continued to rise for about 2 hr. after the introduction of the non-radioactive medium into the chambers. The number of grains over the nucleus did not fall during this period, or fell only slightly; in some experiments there was actually a rise during the first hour. In this respect the connective-tissue cell differed from the macrophage, in which the number of grains over both nucleus and cytoplasm began to fall as soon as the non-radioactive medium was introduced into the chambers. This difference appears to indicate that in the connective-tissue cell there is a pool of acid-soluble RNA precursors which does not rapidly come into equilibrium with exogenous adenosine; if there is such a pool in the macrophage it is presumably very much smaller.

After the second hour in non-radioactive medium the number of grains over the nucleus fell rapidly, the half-life of the process being less than 2 hr. But no turnover of cytoplasmic RNA was observed over the period studied. Although a very slow turnover of cytoplasmic RNA may not have been detected within 10 hr., a turnover of the magnitude observed in the cytoplasm of the macrophage would have been easily measurable. Plate 2*A* shows the radioautograph of a connective-tissue cell fixed after incubation for 20 min. in the radioactive medium. Plate 2*B* shows a cell which was fixed after incubation for 20 min. in the radioactive medium and then 3 hr. in the non-radioactive medium. Plate 2*C* shows a cell which was fixed after incubation for 20 min. in the radioactive medium and then 12 hr. in the non-radioactive medium. For purposes of illustration these radio-

autographs were also exposed for a longer period than was optimum for grain counting.

The curves in Fig. 3 again appear to indicate that most of the nuclear RNA does not pass intact into the cytoplasm. The large rise in the number of grains over the cytoplasm during the first 2 hr. is due to incorporation of labelled material from acid-soluble pools. This rise cannot be due to movement of RNA from nucleus to cytoplasm because during this period the number of grains over the nucleus remains more or less constant; and even if all the labelled RNA in the nucleus had been transferred to the cytoplasm in the first 2 hr. it would still not have accounted for the increase in the number of grains over the cytoplasm. Owing to the complications introduced by the continued labelling of RNA from acid-soluble pools it is not possible to measure the movement of labelled RNA from nucleus to cytoplasm during this initial period. In the following 10 hr., however, there was an average loss of about 35 grains/cell from over the nucleus, but no detectable increase in the number of grains over the cytoplasm. Although the magnitude of the counting errors might have masked an increase of about 10 grains/cell over the cytoplasm, an increase of much more than this would have been detected. It therefore appears that in the connective-tissue cell also at least two-thirds of the nuclear RNA does not pass into the cytoplasm in a stable form.

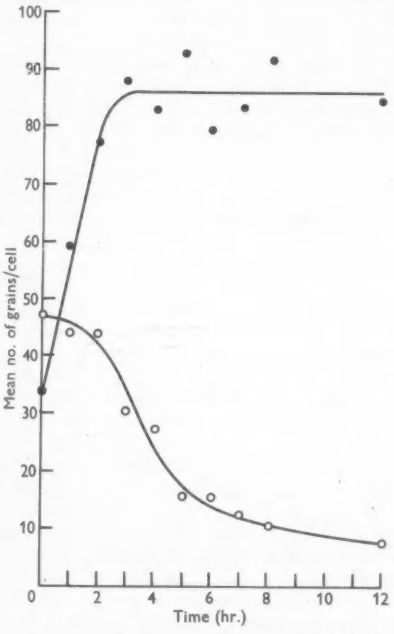

Fig. 3. Turnover of nuclear and cytoplasmic RNA in the connective-tissue cell. ○, Nuclear RNA; ●, cytoplasmic RNA. Cells were exposed to tritium-labelled adenosine and tritium-labelled thymidine for 20 min., washed with three changes of non-radioactive medium containing an excess of unlabelled adenosine and thymidine and then incubated for 12 hr. in non-radioactive medium containing 2 mM-adenosine and 2 mM-guanosine. The incubation in non-radioactive medium was begun at zero time. Cells not in the phase of DNA synthesis were selected for grain counts.

Nucleolar ribonucleic acid

Experiments on the turnover of nucleolar RNA could only be carried out on the connective-tissue cell, since the macrophage has no visible nucleoli. When the connective-tissue cells were fixed after a period of incubation in medium containing tritium-labelled adenosine radioautographs always showed the heaviest labelling over the nucleoli. This is illustrated in Plate 2 *A* and *B*. From radioautographic results of this sort obtained with other cells it has been concluded that the turnover of nucleolar RNA is more rapid than the turnover of RNA elsewhere in the cell (Ficq, 1953, 1955; Vincent, 1955). But the number of grains over any

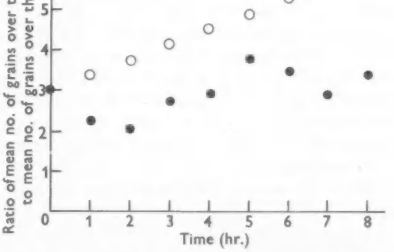

Fig. 4. Rate of turnover of nucleolar RNA in the connective-tissue cell compared with the rate of turnover of RNA in the rest of the nucleus. Cells were exposed to tritium-labelled adenosine and tritium-labelled thymidine for 20 min., washed with three changes of non-radioactive medium containing an excess of unlabelled adenosine and thymidine and then incubated for 8 hr. in non-radioactive medium containing 2 mM-adenosine and 2 mM-guanosine. The incubation in non-radioactive medium was begun at zero time. Cells not in the phase of DNA synthesis were selected for grain counts. ●, Ratio of the mean number of grains over the nucleus to the mean number of grains over the nucleoli; ○, expected change in this ratio if the rate of turnover of nucleolar RNA exceeded the rate of turnover of RNA in the rest of the nucleus by 20%.

part of a cell is a reflexion of the total amount of labelled precursor incorporated at that site. Heavier labelling over the nucleoli may indicate either that the nucleolar RNA is incorporating the precursor more rapidly or merely that there is a greater concentration of RNA at the nucleolus. It is possible to distinguish between these possibilities by measuring the rate at which the labelled precursor is released from the nucleolar RNA.

Cells were incubated for 20 min. in the presence of tritium-labelled adenosine and thymidine, washed with three changes of non-radioactive medium containing an excess of unlabelled adenosine and thymidine and then incubated for 8 hr. in non-radioactive medium containing 2 mM-adenosine and 2 mM-guanosine. At the end of the period of incubation in radioactive medium and at hourly intervals afterwards the cells from one of the chambers were fixed and radioautographs were prepared in the usual way. Cells not in the phase of DNA synthesis were selected and grain counts made over the nucleoli and over the rest of the nucleus. The results are shown in Fig. 4. Over 8 hr. there appeared to be no significant change in the ratio of the number of grains over the nucleoli to the number over the rest of the nucleus. If the turnover of nucleolar RNA had been either faster or slower than the turnover in the rest of the nucleus, a change in this ratio would have been observed. The open circles in Fig. 4 show the expected change in this ratio if the nucleolar RNA had turned over 20% more quickly than the RNA in the rest of the nucleus. This slope was calculated from the observation that the fall in the number of grains over both nucleus and nucleoli was, to a first approximation, an exponential process and that this process in the nucleus had a half-life of 2 hr. or less. It appears therefore that the rate of turnover of nucleolar RNA does not differ greatly, if at all, from the rate of turnover of RNA in the rest of the nucleus. The heavy labelling over nucleoli observed in radioautographs is thus to be explained by a greater concentration of RNA at these sites.

Turnover of cytidine

Similar experiments were carried out with tritium-labelled cytidine as the precursor. The rates of turnover of nuclear, nucleolar and cytoplasmic RNA as measured by the release of labelled cytidine were essentially similar to those obtained with labelled adenosine. In one respect, however, the results obtained with cytidine differed from those obtained with adenosine. In radioautographs of connective-tissue cells which had been incubated in the presence of tritium-labelled adenosine the ratio of the number of grains over the nucleoli to the number over the rest of the nucleus was about 1:3. In radioautographs of cells which had been incubated in the presence of tritium-labelled cytidine, this ratio was about 1:1·4. Since the rates of turnover of adenosine and cytidine in both nuclear and nucleolar RNA were essentially the same, this difference appears to indicate that the ratio of adenine to cytosine in the nucleolar RNA is substantially lower than the ratio of adenine to cytosine in the RNA of the rest of the nucleus.

Further observations on the role of the nucleolus in the initiation of deoxyribonucleic acid synthesis

In a previous paper (Harris, 1959) radioautographic evidence was produced in support of the view that the synthesis of DNA was initiated at the nucleolus. When connective-tissue cells in the phase of DNA synthesis were exposed for a short period to tritium-labelled thymidine their nuclei showed three patterns of labelling: (i) labelling over the nucleoli only; (ii) labelling over the nucleoli together with labelling over the rest of the nucleus; (iii) labelling over the rest of the nucleus without labelling over the nucleoli (Plate 1, Harris, 1959). It was found that the phases showing nucleolar labelling preceded the phase showing no nucleolar labelling. During mitosis, however, when the nucleoli are no longer present as discrete structures, all the labelled thymidine which has been incorporated is found to be associated with the chromosomes. It may therefore be concluded that when a cell shows nucleolar labelling after exposure to tritium-labelled thymidine the thymidine has actually been incorporated into chromatin which is intimately associated with the nucleolus. The experiments now to be described were designed to determine whether the chromatin associated with the nucleoli is a specific and constant part of the chromosomal material, as appears to be the case in certain plants (McClintock, 1934), or whether the association between the nucleoli and the chromatin is random.

Cultures of connective-tissue cells were incubated for 15 min. in medium containing tritium-labelled thymidine. The cells were then washed with three changes of non-radioactive medium containing an excess of unlabelled thymidine and incubated for a further 10 hr. in non-radioactive medium containing no added nucleic acid precursors. The cultures were then fixed and radioautographs made in the usual way. Many of the cells which were in the phase of DNA synthesis at the time of exposure to the labelled thymidine had completed mitosis during the 10 hr. period (Harris, 1959). For some time after mitosis it is possible to recognize the two daughter cells by their relatively small size, their proximity or contiguity and their similar morphology. The preparations were scanned for recognizable pairs of daughter cells and the pattern of nuclear and nucleolar labelling in these cells was observed.

Of fifty pairs of daughter cells which were examined, forty-two showed a similar pattern of labelling in both members of the pair, that is, both members showed either nucleolar labelling only, or nucleolar labelling together with labelling over the rest of the nucleus or labelling over the rest of the nucleus without labelling over the nucleoli. In eight pairs of daughter cells the pattern of labelling was not clear enough to establish similarity or dissimilarity, but not one pair was found in which one member clearly showed a different pattern of labelling from the other. These results appear to rule out the possibility that the association between the nucleoli and the chromatin is a random one. But they do not necessarily show that the pattern of labelling in the two daughter cells was similar to the pattern of labelling in the cell from which they were derived. This question was resolved in the following way.

It has previously been shown that when growing connective-tissue cells are dissociated by treatment with trypsin and then introduced into chambers most of them are not in the phase of DNA synthesis immediately after treatment with trypsin but enter this phase during the next 24 hr. (Harris, 1959). As increasing numbers of cells pass into the phase of DNA synthesis the percentage of labelled cells showing nucleolar labelling after a short exposure to tritium-labelled thymidine rises. Five pairs of partially synchronized cultures were prepared by treatment with trypsin in this way and, 12 hr. after introduction of the dissociated cells into the chambers, one pair of cultures was exposed for 15 min. to medium containing tritium-labelled thymidine. One culture from this pair was fixed at the end of the 15 min. period. The other was washed with three changes of non-radioactive medium containing an excess of unlabelled thymidine, incubated for 24 hr. in non-radioactive medium containing no nucleic acid precursors and then fixed. At 15, 18, 21 and 24 hr. after introduction of the cells into the chambers one pair of cultures was treated in the same way. Almost all the cells which were in the phase of DNA synthesis at the time of exposure to the labelled thymidine would have completed mitosis within 24 hr. under these conditions (Harris, 1959), so that the labelled cells in the cultures fixed after incubation for 24 hr. in the non-radioactive medium corresponded to the daughter cells of the labelled cells in the cultures fixed immediately after incubation in the radioactive medium. The percentage of labelled cells showing nucleolar labelling in the two groups is shown in Fig. 5. It will be seen that the rise in this percentage in the group of cultures fixed immediately after incubation in the radioactive medium is mirrored in the group fixed 24 hr. later. This indicates that the pattern of labelling in the parent cells is reproduced in the daughter cells. This finding, together with the observation that the two members of a pair of daughter cells have the same pattern of labelling, appears to establish the fact that the DNA associated with the nucleoli is a specific and constant part of the chromosomal material. It is apparently in this part of the chromosomal material that the replication of DNA begins.

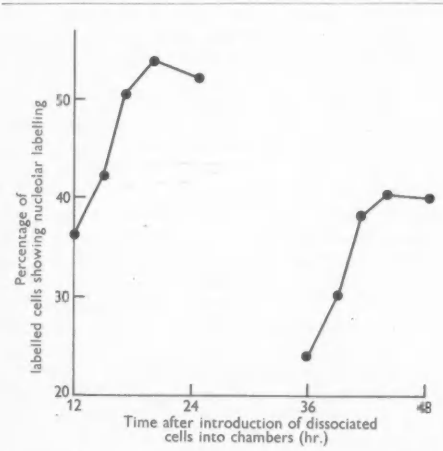

Fig. 5. Pattern of nuclear and nucleolar labelling in partially synchronized cultures of connective-tissue cells exposed for short periods to tritium-labelled thymidine. The curve on the left illustrates the rise in the percentage of labelled cells showing nucleolar labelling which takes place as increasing numbers of cells pass into the phase of DNA synthesis. These cultures were fixed immediately after exposure to the labelled thymidine. The curve on the right shows a corresponding rise in cultures exposed to the labelled thymidine at the same time as the first group, but fixed after further incubation for 24 hr. in non-radioactive medium.

DISCUSSION

The results of the present investigation are difficult to reconcile with some current views about the functions of nuclear and nucleolar RNA. Several investigators have shown in animal experiments that certain precursors are incorporated more rapidly into nuclear than into cytoplasmic RNA (for review of literature see Smellie, 1955). But in animal experiments it is seldom possible to measure to what extent turnover is the result of concomitant cell multiplication and cell death, and the observed rate of turnover is also greatly influenced by the fact that the products of turnover are re-utilized. Interpretation of the data under such conditions is difficult.

Barnum, Huseby & Vermund (1953) and Smellie, McIndoe, Logan, Davidson & Dawson (1953) obtained results in animal experiments which were difficult to reconcile with the view that nuclear RNA was a precursor of cytoplasmic RNA, but the possibility could not be directly excluded. The curves obtained in the present experiments appear to be incompatible with the view that nuclear RNA can be a precursor of cytoplasmic RNA. It appears that in the intact cell only a small fraction of the nuclear RNA can pass into the cytoplasm in a stable form. If nuclear RNA is thought to function as a macromolecular vehicle carrying 'information' from the genes to the cytoplasm, the present findings indicate that during the greater part of the life of the cell most of the nuclear RNA is not engaged in this function. The significance of the rapid turnover of nuclear RNA remains obscure; it does not, however, appear to be directly related to cell multiplication since it occurs in both multiplying and non-multiplying cells.

It is tempting to suppose that the difference between the connective-tissue cell and the macrophage in the observed turnover rate of cytoplasmic RNA represents an essential difference between a multiplying and a non-multiplying cell. But the apparent absence of cytoplasmic turnover in the connective-tissue cell must be regarded with caution. The fact that this cell contains a large pool of nucleic acid precursors may mean that labelled intermediates of cytoplasmic RNA turnover are less effectively diluted by unlabelled nucleosides in this cell than in the macrophage, where such a pool, if it exists at all, is much smaller. And even if the difference in the observed rates of cytoplasmic turnover does reflect a difference in the true rates of turnover, this difference may have little connexion with the fact that one of the cells multiplies and the other does not.

The present experiments show that in the connective-tissue cell nucleolar RNA has much the same turnover rate as the RNA in the rest of the nucleus. Any theories of nucleolar function which involve the notion of a more rapid turnover of RNA at the nucleolus than elsewhere can therefore hardly apply to this cell. While there is some indication that the base composition of the nucleolar RNA is different from the base composition of the RNA in the rest of the nucleus, this difference does not necessarily imply a difference in function. It is possible that different parts of the RNA in the rest of the nucleus also have different base compositions. In assessing the significance of the higher concentration of RNA at the nucleolus, it should be remembered that there is also a higher concentration of DNA intimately associated with it, and that the structure itself consists of an extremely high concentration of protein (Dounce, 1955). The function of the nucleoli remains obscure; but it is difficult to suppose that this function is unrelated to the observation that it is in the chromatin associated with the nucleoli that the reduplication of DNA is initiated.

SUMMARY

1. Quantitative radioautographic techniques were used to measure the turnover of ribonucleic acid in the nucleus and cytoplasm of two animal cells: the connective-tissue cell, which multiplies *in vitro*, and the macrophage, which does not multiply but remains alive for many hours.

2. Tritium-labelled adenosine and cytidine were used as the precursors and turnover was measured by the rate of release of these precursors from the ribonucleic acid.

3. In both cells there was a rapid turnover of nuclear ribonucleic acid. The observed half-life of the nuclear ribonucleic acid involved in turnover was less than 2 hr. In the macrophage there was also a turnover of cytoplasmic ribonucleic acid; the observed half-life of this ribonucleic acid was 5–7 hr. No turnover of cytoplasmic ribonucleic acid could be detected in the connective-tissue cell over the period studied.

4. The results in both cells appeared to be incompatible with the view that the nuclear ribonucleic acid was a precursor of the cytoplasmic ribonucleic acid.

5. In the macrophage the findings indicated that only a small proportion, if any, of the nuclear ribonucleic acid could have passed into the cytoplasm during the course of the experiments, unless the nuclear ribonucleic acid were rapidly destroyed on entering the cytoplasm. In the connective-tissue cell the magnitude of the counting errors might have permitted more of the nuclear ribonucleic acid to pass into the cytoplasm without being detected; but, even so, it could be shown that in this cell also most of the nuclear ribonucleic acid did not pass into the cytoplasm in a stable form.

6. The observed rate of turnover of nucleolar ribonucleic acid was not significantly different from the observed rate of turnover of the ribonucleic acid in the rest of the nucleus. There was, however, a higher concentration of ribonucleic acid in the region of the nucleoli and there was indirect evidence suggesting that the base composition of this ribonucleic acid was different from that of the ribonucleic acid in the rest of the nucleus.

7. The chromatin associated with the nucleoli represents a specific part of the chromosomal material which maintains its association with the nucleoli from one cell generation to the next. It is apparently in this chromatin that the reduplication of chromosomal deoxyribonucleic acid is initiated.

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Intermediate Reactions in Protein Synthesis by the Isolated Cytoplasmic-Membrane Fraction of *Bacillus megaterium*

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Studies of the uptake of labelled amino acids into the various cellular fractions of *Bacillus megaterium* have shown that the primary site of protein synthesis in this organism is located in the fraction which sediments when broken protoplasts are centrifuged at 10 000 *g*. The activity probably resides in the principal component of this fraction, which is the cytoplasmic membrane (Hunter, Crathorn & Butler, 1957; Butler, Crathorn & Hunter, 1958). This may hold true for bacteria in general, as similar findings have now been reported for *Bacillus subtilis* (Nomura, Hosoda & Nishimura, 1958) and *Escherichia coli* (Spiegelman, 1959).

It was further shown (Butler *et al.* 1958) that the isolated cytoplasmic-membrane fraction was capable of incorporating labelled amino acids into proteinaceous material at rates comparable with those of the intact protoplast. Two aspects of the role of the isolated membrane system are considered here: (1) the relationship between normal protein synthesis in the whole cell and the incorporation of labelled amino acids into the cell-free system; (2) the incorporation process itself, with a view to identifying intermediate reactions. As previous studies of protein synthesis in other systems (Hoagland, Keller & Zamecnik, 1956; Hoagland, Stephenson, Hecht & Zamecnik, 1958; Nismann, Bergmann & Berg, 1957; De Moss & Novelli, 1955) have demonstrated the probable importance of amino acid-activating enzymes and of ribonucleic acid as factors controlling the course of amino acid incorporation into protein (so that peptides containing the amino acids can subsequently be obtained), particular emphasis has been laid on the investigation of their roles in this system.

A preliminary account of some of this work has already been given (Brookes, Crathorn & Hunter, 1959).

METHODS

Organism. The strain of *B. megaterium* (originally KM) was cultured as previously described (Butler *et al.* 1958). For all the experiments described here the organism was grown at 30° in a glucose–salts medium (C medium; McQuillen, 1955).

Preparation of subcellular fractions. It was found that conversion of whole cells into protoplasts was best effected in a medium containing 0·02% of lysozyme and 0·5M-KH_2PO_4, brought to pH 7·0 with NaOH. The lysozyme treatment was continued until a microscopic examination of a sample failed to reveal the presence of more than an occasional whole cell. The upper limit for whole-cell content was about 0·01%, although in many experiments not a single whole cell was observed in the protoplast preparations. Before carrying out any biochemical experiments, the protoplasts were usually aerated at 30° for 1 hr. in C medium containing, in addition, 0·5M-KH_2PO_4 and 1% of glucose, the whole brought to pH 7·0 with NaOH. Lysis of the protoplasts was effected by diluting this medium with

1–2 vol. of water and shaking by hand until microscopic examination showed that no protoplasts remained intact. The cytoplasmic-membrane fraction was then collected by centrifuging at 10 000–20 000 *g* for 10–20 min.

Experiments with the isolated membrane fraction. Where the uptake of labelled amino acids was being studied, the cytoplasmic-membrane fraction was resuspended in C medium containing 0·5 M-KH_2PO_4, the whole being brought to pH 7·0 with NaOH (C–phosphate). In short-term experiments, the presence of glucose had no effect on the system, but the addition of the 0·5 M-KH_2PO_4 to the medium gave more reproducible results than the C–glucose used in earlier experiments (Butler *et al.* 1958). Many other media were tried, but all of these inactivated the amino acid-incorporation system to some extent, usually completely.

Further fractionation was effected by suspending membranes in water (25–100 mg./ml.) and subjecting the suspension to ultrasonic vibrations from a small MSE Mullard tissue disintegrator until it was completely homogeneous in appearance to the naked eye. Throughout this treatment, which usually lasted about 10 min., the tube containing the suspension was surrounded by a cooling bath so that the mean temperature in the tube did not rise above 30°. The sedimentable and 'soluble' fractions produced by this treatment were separated by centrifuging at 100 000 *g* for 5 min., and the ultrasonic treatment was repeated on the sedimented fraction. Homogeneity was more rapidly attained the second time. The homogenate was again centrifuged at 100 000 *g* and the 'soluble' fractions from the two successive treatments were combined.

Digestions with ribonuclease, lysozyme, histone and protamine (all at 1 mg./ml.) were carried out in C–phosphate, phosphate buffer or water.

Chloramphenicol was used at 100 μg./ml., and its inhibiting action was found to be complete after incubation at 30° for 15 min.

Digestions with 0·1 N-NaOH were carried out at 30°.

Isolation of ribonucleic acid. Separation of the ribonucleic acid (RNA) from the 'soluble' fraction was effected by treatment of this fraction with an equal volume of aq. 90% phenol (Kirby, 1956). From the upper layer of this mixture phenol was extracted with ether. The aqueous extract was then brought to 2% with potassium acetate before precipitating the RNA with 2 vol. of ethanol. Contaminating carbohydrate was removed by extracting the RNA into 2-methoxyethanol (Kirby, 1956). After dialysis and drying in a vacuum desiccator, the RNA was obtained as an amorphous white powder. Some of the contaminating deoxyribonucleic acid (DNA) could be removed by dialysis against C medium (McQuillen, 1955) in the presence of deoxyribonuclease (10 μg./mg. of RNA), the purified RNA being recovered by precipitation with ethanol.

Protein solubilized in the course of the phenol extraction could be precipitated from the lower liquid phase by adding ether, removing the ether layer, sedimenting the precipitated protein and finally washing with ether.

Experiments on amino acid activation. These experiments were carried out under conditions similar to those used by Nismann *et al.* (1957). The medium contained 0·1 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris), 5 mM-$MgCl_2$, 2 mM-adenosine triphosphate (ATP) and 5 mM-NaF. Solutions of amino acids to be examined were added to give a final concn. of 5 mM and the enzyme preparation was added to give 80 μg. of protein/ml. When the enzymic activities of cell cytoplasm or ultrasonically fractionated membranes were being investigated, they were prepared in the tris buffer as described above.

Incubations were carried out at 37° for 1 hr., and the ATP was finally isolated by absorption on charcoal as described by Crane & Lipmann (1953). The washed charcoal was finally boiled for 10 min. with N-HCl and the radioactivity of the hydrolysate was determined.

Extraction of lipid fractions from membranes. Extractions with deoxycholate were carried out in a medium containing glycylglycine (2·64%), NaCl (1·17%) and sodium deoxycholate (0·5%), the whole being brought to pH 7·8 with NaOH. Membranes previously labelled with [^{14}C]amino acids were shaken in this medium (10 mg./ml.) at 37° for 20 min., followed by standing for 12 hr. at 0°. This procedure solubilized about 10% of the protein, the remaining 90% being removed by sedimenting at 20 000 *g*.

Extractions with methanol were carried out by boiling the appropriate fraction in the alcohol for 30 min. This same procedure was used whether the material extracted consisted of whole or fractionated membranes, proteinaceous fractions precipitated by trichloroacetic acid or material precipitated by the addition of solvents to phenol extracts.

Digestions with lipase were carried out under conditions similar to those used by Spiegelman, Aronson & Fitz-James (1958) when preparing 'nuclear bodies' from protoplasts. The membranes were digested at 30° at a concentration of 5–10 mg./ml.

Analysis of ^{14}C-labelled proteins. Samples of labelled protein (10–25 mg., containing 0·5–5·0 μc of ^{14}C/g.) from which lipids and nucleic acids had been removed as described previously (Crathorn & Hunter, 1957) were digested at 110° with 6N-HCl (0·2–0·5 ml.) for 18 hr. The HCl was removed, and about 10% of the sample was subjected to upward-flow chromatography on Whatman no. 1 paper in a solvent system containing methyl ethyl ketone–acetic acid–water (3:1:1, by vol.). Amino acid 'markers' were detected with a ninhydrin spray, and the papers were then placed in contact with X-ray film in a light-tight container for 2–4 weeks, and radioautographs were obtained in the usual way.

Partial hydrolyses of labelled samples (10–25 mg. at 2–5 μc of ^{14}C/g.) were carried out for 2 days at 37° in 12N-HCl (0·2–0·5 ml.). The acid was removed *in vacuo* over KOH and the residue dissolved in water (0·1 ml.). About one-half of the sample was subjected to two-dimensional paper chromatography (downward flow) in the following solvent systems: butanol–ethanol–propionic acid–water (10:5:2:5, by vol.) (solvent 1); butanol–acetone–di*cyclo*hexylamine–water (10:10:2:5, by vol.) (solvent 2). Papers were dried, sprayed with ninhydrin and radioautographs prepared as before, except that a longer time was generally required for their adequate exposure (4–8 weeks).

Assays of radioactivity. Protein and other solid samples were prepared for radioactive assay as described previously (Butler *et al.* 1958; Crathorn & Hunter, 1957). Assays of solid samples were carried out by end-window counting as described by Butler *et al.* (1958).

^{32}P samples were counted in 10 ml. samples in an annular liquid counter type M6M (supplied by 20th Century Electronics Ltd., New Addington, Surrey).

Table 1. *Effects of varying concentrations of suspended membranes on the uptake of* L-[^{14}C]*valine into protein fraction*

Membranes (100 mg.) were suspended in C–phosphate and shaken in 500 ml. flasks at 30° for 30 min. before addition of 2 μc of L-[^{14}C]valine. The incubation was continued in the same way for 20 min., and then brought to a close by adding aq. 30% (w/v) trichloroacetic acid to a final concn. of 5%. Other experimental details were as described in the text.

Concn. of suspended membrane (mg./ml.)	Specific radioactivity of protein (μc/g.)
1	0·20
5	0·45
20	0·12

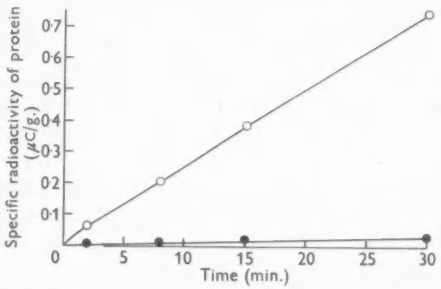

Fig. 1. Inhibition by chloramphenicol (100 μg./ml.) of the incorporation of [^{14}C]amino acids into the protein of the membrane fraction. Membrane samples (50 mg.) suspended in 'C–phosphate' (10 ml.) were shaken with chloramphenicol (100 μg./ml.) at 30° for 15 min. [1-^{14}C]-Glycine (0·2 μc) was then added to each sample and the incubations were continued for varying times before adding trichloroacetic acid. ○, Controls; ●, chloramphenicol present.

Estimation of nucleic acid. RNA was determined by the orcinol method (Cerriotti, 1955) and DNA by Cerriotti's (1952) indole method.

Materials. Generally-labelled L-amino acids (5–9 μc/μmole) and sodium [^{32}P]pyrophosphate were supplied by The Radiochemical Centre, Amersham, Bucks. [1-^{14}C]-Glycine (10·3 μc/mg.) was synthesized in these Laboratories. All the [^{14}C]amino acids were dissolved in water at 10 μc/ml. and used without any dilution with unlabelled amino acids, unless otherwise stated.

Lipase was supplied by the Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.; it was partially purified by the method of Spiegelman *et al.* (1958).

RESULTS

Experiments on the uptake of [^{14}C]*amino acids by the whole-membrane fraction.* Additional experiments (see also Butler *et al.* 1958) have enabled us to define more precisely the conditions required for maximum uptake of labelled amino acids. An improved medium has been developed and, further, it was found (Table 1) that maximum uptake of label into protein was achieved if the membranes were suspended in the medium at a concentration of 5 mg./ml. It was also found that the rates of amino acid incorporation were slightly enhanced if the membranes were thoroughly dispersed by shaking in a Mickle disintegrator rather than by hand. But the small advantage gained in this way was hardly sufficient to offset the extra manipulations involved.

The rates of uptake of different labelled amino acids have also been found to vary considerably, but as no information is available on the concentrations of amino acids present, both free and within the proteins, it has not been possible to make a quantitative assessment of these results.

Experiments with inhibitors. The effect of chloramphenicol on the incorporation system is illustrated in Fig. 1. After incubation for 30 min. in the presence of [1-^{14}C]glycine, the treated samples showed an inhibition of 96% in the uptake of the labelled amino acid into the protein fraction. In a corresponding experiment with whole cells carried out at the same time, the uptake of [1-^{14}C]glycine into the fraction precipitable by trichloroacetic acid was five times greater than that in the membrane experiment, but the inhibition produced by the same concentration of chloramphenicol was again 96%.

Table 2 shows the effect produced on the incorporation system by pre-incubating the membranes with ribonuclease and various basic proteins.

Table 2. *Effect of pre-incubating the membrane fraction with ribonuclease and other proteins on the amino acid-incorporation system*

For each treatment, membranes (50 mg.) were suspended in C–phosphate (10 ml.) containing the dissolved protein (10 mg.). After shaking at 30° for 1 hr., L-[^{14}C]lysine (2 μc each) was added to one-half of the reaction flasks and the incubations were continued for a further 30 min. The membranes were sedimented and resuspended in fresh medium before L-[^{14}C]lysine was added to the other flasks. The incubations were terminated and the protein samples obtained as described in the text.

Protein added	Specific radioactivity of membrane protein (μc/g.)	
	Original medium	Fresh medium
—	0·52	0·63
Ribonuclease	0·033	0·29
Lysozyme	0·050	0·32
Histone	0·012	0·053
Protamine	0·008	0·112

The reversible nature of the inhibition produced by ribonuclease under conditions where it is enzymically active, and the similarity to that produced, in particular, by lysozyme would suggest that it is not a consequence of its action as an enzyme.

Total and partial acid hydrolysis of labelled protein from membranes. Total hydrolyses were carried out on protein samples derived from membranes that had been labelled with [1-^{14}C]glycine, L-[^{14}C]lysine and L-[^{14}C]valine. In each case, radioautographs of the hydrolysates showed that the original amino acid added was the only detectable radioactive compound present. However, other compounds containing less than 10% of this radioactivity might have remained undetected. Partial acid hydrolyses were carried out on membranes labelled with L-[^{14}C]leucine, L-[^{14}C]valine and L-[^{14}C]-alanine. In each case the radioautographs showed that several labelled peptides were present in the hydrolysates. The original amino acid was also present, but there appeared to be a larger proportion of the label in the peptide spots (Fig. 2).

Experiments with ultrasonically disrupted membranes. When membranes labelled for 20–30 min. were disrupted ultrasonically, it was found that the protein of the sedimented fraction had about one-sixth of the specific radioactivity of the protein of the 'soluble' fraction. Thus membranes (0·5 g.) labelled for 30 min. with L-[^{14}C]valine (10 μc) in the usual way gave 'soluble' protein with a specific radioactivity of 2·6 μc/g. and sedimentable protein with a specific radioactivity of 0·41 μc/g. When the labelling time was reduced, the specific radioactivity of the sedimentable protein was relatively higher; indeed, when the time was only 2 min. the two protein fractions had comparable specific radioactivities.

The ultrasonic treatment was also carried out on unlabelled membranes suspended in C–phosphate, and the fractions obtained were separately incubated with L-[^{14}C]alanine. As shown in Table 3, only the sedimentable protein could be labelled directly, although the level of labelling obtained was much lower than that attained with whole membranes.

Experiments on amino acid activation. Initial experiments were performed on whole membranes, rather variable results being obtained. Exchange of ^{32}P between [^{32}P]pyrophosphate and ATP was, however, found to take place at rates 100–300% above control values with the following amino acids: L-leucine, L-proline, L-asparagine, L-cysteine, L-lysine and L-histidine. Chloramphenicol at 100 μg./ml. had no effect on the process. No significant enzymic activities were observed when equivalent amounts of cytoplasm were used.

However, when the membranes were suspended in the tris buffer used for the ^{32}P-exchange experiments and disintegrated ultrasonically, it was

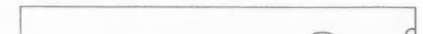

Fig. 2. Tracing of radioautograph prepared from two-dimensional paper chromatogram of partial acid hydrolysate of protein from membrane fraction labelled with L-alanine. All the operations were carried out as described in the text. All the areas outlined represent peptides, apart from the L-alanine spot itself.

Table 3. *Separate labelling of fractions derived ultrasonically from whole membranes*

Membranes were fractionated as described in the text. Fractions (50 mg.) were adjusted to 5 mg./ml. in C–phosphate and then shaken at 30° with L-[^{14}C]alanine (2 μc) for 30 min., before adding trichloroacetic acid to a final concentration of 5%.

Fraction	Specific radioactivity of protein (μc/g.)
Whole membranes	5·0
Sedimented fraction	0·11
'Soluble' fraction	0·005

Table 4. *Transfer of ^{32}P from sodium [^{32}P]pyrophosphate to adenosine triphosphate in the presence of the 'soluble' fraction of ultrasonically disintegrated membranes*

Preparation of the 'soluble' fraction and the conditions of the ^{32}P-exchange reaction were as described in the text. For each treatment, 0·25 μc of sodium [^{32}P]pyrophosphate (specific activity 6·40 mc/m-mole) was used in a total volume of 2 ml.

Treatment	Percentage transfer of ^{32}P to ATP
Control, without 'soluble' fraction	0·4
Control, without added amino acid	1·9
Control, without added ATP	0·8
L-Asparagine	6·0
L-Cysteine	11·0
L-Histidine	6·3
L-Leucine	14·8
L-Lysine	11·0

found that the amino acid-activation enzymes were liberated into the 'soluble' fraction. All the amino acids studied stimulated the rate of ^{32}P-exchange between pyrophosphate and ATP several times above the control values (Table 4). Very little of the enzymic activity resided in the sedimented fraction.

Properties of ribonucleic acid from the membrane fraction. In a typical experiment, 2·5 g. of whole cells were lysed in the usual way and phenol extraction of the ultrasonically disrupted membrane fraction was carried out as described in the Methods section. The crude RNA had $\lambda_{max.}$ 260, $E^{1\%}_{1cm.}$ 30, which after purification gave 30 mg. of solid having $\lambda_{max.}$ 260, $E^{1\%}_{1cm.}$ 210. The final product was completely soluble in hot trichloroacetic acid. Analyses indicated that the material was about 70% of RNA and 30% of DNA. After treatment with deoxyribonuclease, the RNA content rose to 87% (colorimetric analysis). The product contained 6·2% of P, and a base ratio determination (Wyatt, 1951) showed that thymine was absent.

Where the membranes had been pre-incubated with [^{14}C]amino acids, the RNA obtained carried a radioactive label. The amount of the label varied widely from one experiment to another. Thus when 500 mg. of membranes were incubated for 20 min. with 10 μc of labelled amino acids under the usual conditions, the specific radioactivities of the purified RNA obtained in six different experiments varied from 0·03 to 5·0 μc/g. The yields of RNA obtained also varied between 5 and 25 mg., usually being less than the yield obtained when freshly prepared membranes were extracted immediately without any incubation at 30°.

The yields of RNA (by wt.), as well as the degree of labelling, bore no obvious relation to the specific radioactivity of the protein obtained in the same experiments. But this may be due to technical deficiencies in the method of RNA extraction. Table 5 shows the results of one such experiment in which the final specific radioactivity of the protein was 0·6 μc/g.

Table 5. *Labelling of membrane ribonucleic acid with* L-[^{14}C]*alanine*

The membranes (540 mg.) were incubated as described in the text with 10 μc of L-[^{14}C]alanine. One-third of the suspension was withdrawn at each of the indicated times, cooled rapidly to 0° and further treated as described in the text.

Incubation time (min.)	Specific radioactivity of RNA (μc/g.)
2·5	0·018
7·5	0·16
20	0·48

It does not seem likely that any appreciable quantity of the label attached to the RNA can be ascribed to metabolic conversion of the added amino acids into purines or pyrimidines. Thus labelled RNA (0·12 μc/g.) obtained in one experiment where L-[^{14}C]arginine was used was subjected to acid hydrolysis. 'Carrier' unlabelled L-arginine was added to the hydrolysate and found to retain the bulk of the ^{14}C label on recrystallization. However, the purified samples of RNA appeared to be free from labelled protein: precipitation with cold trichloroacetic acid in the presence of a large excess of unlabelled protein, followed by heating with hot trichloroacetic acid, left a residue that contained no radioactivity. On the other hand, the radioactivity could readily be removed by mild treatments at alkaline pH. Thus labelled RNA (0·2 μc/g.) derived from membranes incubated with L-[^{14}C]valine lost 30% of its radioactivity when left at room temperature for 10 min. in 0·02N-KOH, and 90% if the temperature was raised to 30° for the same period, the RNA being recovered in each case by precipitation with ethanol. Of the radioactivity, 75% was removed by treatment with ribonuclease (1 mg./ml.) for 1 hr. at 30°. Treatment with deoxyribonuclease, on the other hand, had no effect.

In one experiment where labelled RNA (10 mg., 0·2 μc/g.) was incubated for 10 min. in C–phosphate (10 ml.) with the sedimented fraction (10 mg.) derived from ultrasonically treated membranes, 37% of the label was transferred to material insoluble in hot trichloroacetic acid. Guanosine triphosphate (0·1 mg./ml.) was added to the normal C–phosphate medium. Attempts to repeat this experiment have been successful in only one out of four, and similar 'transfer' experiments carried out with whole membranes have also given negative results. However, a 10% transfer of the label from RNA to the protein of whole protoplasts has been effected, and experiments are being continued.

Experiments suggesting a role for lipids on protein synthesis. Treatment of labelled membranes with sodium deoxycholate led to the solubilization of about 10% of the protein. The radioactive materials in both soluble and insoluble fractions were still precipitable by hot trichloroacetic acid, but, as shown in Fig. 3, the specific activity of the protein in the extracted lipoprotein fraction was much the higher, whatever the length of time for which the membranes were labelled.

This result led us to examine the action of lipase on labelled membranes. Many experiments were performed, with somewhat variable results, but it was always found that incubation in the presence of lipase resulted in a rapid loss of radioactivity from the fraction insoluble in hot trichloroacetic

acid. This is illustrated in Fig. 4. In the course of the same experiment, the weight of material recovered in the protein fraction dropped to 50% of the control in the first 5 min. of the lipase treatment, but thereafter fell very little more in 55 min. In further experiments it was found that a greater percentage of the radioactivity could be removed from membranes labelled for short periods than for periods of 30 min. or more.

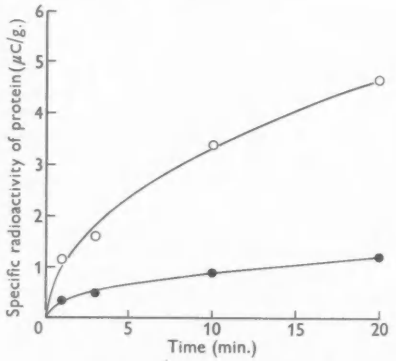

Fig. 3. Deoxycholate fractionation of membrane protein labelled with L-[^{14}C]leucine. The membrane fraction was labelled for varying times as described in the text. ●, Residue from extraction with sodium deoxycholate; ○, fraction extracted during treatment with sodium deoxycholate.

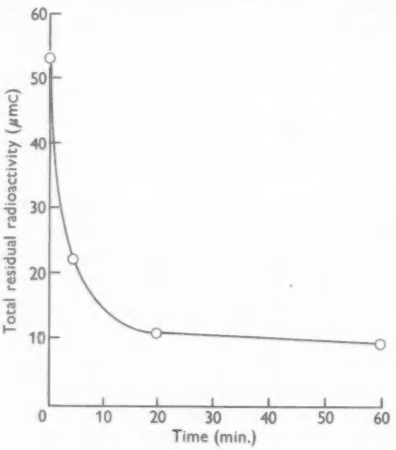

Fig. 4. Rate of removal of radioactivity by lipase from the protein fractions of membranes labelled in the usual way with L-[^{14}C]lysine. In each treatment, membranes (40 mg.) were treated with crude lipase (10 mg.) under the conditions described in the text.

It was also found that incubation of labelled membranes with 0·1N-NaOH at 30° for 10 min. removed between 10 and 50% of the protein-bound radioactivity.

Finally, the extraction of labelled membranes with boiling methanol led to the removal of considerable amounts of radioactivity in a form no longer precipitable with trichloroacetic acid. The bulk of the methanol-soluble material was present in the part of the membrane fraction which was still sedimentable after the ultrasonic treatment (see above). As shown in Table 6, about 62% of the label in this fraction was extracted into methanol. Preliminary chromatographic results indicate that the material extracted is not in the form of the free amino acid; its composition is probably complex and it is being examined in greater detail.

However, the whole of the label extracted in this and in the other experiments described in this section cannot be attributed to common lipid constituents formed by metabolic processes from the amino acids. Extraction with lipid solvents at temperatures below 40°, although it solubilizes much of the lipid, fails to lower the specific radioactivity of the protein; the label is therefore not present in the common lipids. Only treatments such as boiling with methanol, which is reported to disrupt lipoprotein complexes (Reichert, 1944), liberates the label and it might be inferred that the label is present in such a form. Furthermore, since total hydrolysis of the whole membrane fraction enabled the bulk of the activity to be recovered in the form of the original amino acid, it can be concluded that the methanol extract also contains the amino acid residue in some form.

DISCUSSION

The present investigation has shown that the isolated cytoplasmic-membrane fraction of *B. megaterium* is capable of incorporating labelled amino acids into peptides. Since (1), the incorporated [^{14}C]amino acid can be retrieved unchanged from total hydrolysates of the labelled-protein fractions under conditions where no other [^{14}C]-compound is detected, and (2) numerous labelled

Table 6. *Removal of bound radioactivity from membrane fractions by extraction with methanol*

Membranes (500 mg.) labelled with L-[^{14}C]phenylalanine (10 μc) for 15 min. were fractionated as described in the text.

Fraction	Specific radioactivity of protein (μc/g.) Before methanol extraction	After methanol extraction
Sedimented	0·20	0·074
'Soluble'	0·52	0·51

peptides are present in partial hydrolysates of [^{14}C]protein, it is concluded that some of the amino acid must have been bound in peptide form, and probably in large peptides or proteins. The fact that the incorporation process is affected by chloramphenicol to the same extent as protein synthesis in intact cells further indicates that the normal mechanisms of protein synthesis are operating in the isolated membranes. Although we cannot completely rule out the presence of whole cells in all our membrane preparations, they were not observed in several experiments where fully active membrane preparations were obtained. In any case, the maximum contamination of 0·01 % would appear to be grossly inadequate to account for the incorporations of labelled amino acids that have been observed.

With the procedure adopted for extracting, washing and assaying the radioactivities of protein fractions, adsorption errors appear to be very small. If labelled-protein samples are dialysed against aqueous solutions containing large amounts of the corresponding unlabelled amino acid before or after the addition of trichloroacetic acid, the final counts obtained are not significantly altered. For a time, precipitations with trichloroacetic acid were carried out in the presence of excess of the unlabelled amino acid, but as several controlled experiments showed that this procedure, also, made no significant difference it was discontinued.

The membrane fraction appears to contain all of the amino acid-activating enzymes of the cell, and these are liberated in a soluble form by ultrasonic treatment. It is possible that activation enzymes for all the common amino acids are present, as in the *Escherichia coli* preparation of Nismann *et al.* (1957).

The successful isolation of RNA from the membrane fraction suggests that Weibull & Bergström (1958) may be wrong to regard as insignificant the 1–2 % of RNA that they detected in the cytoplasmic membrane of *B. megaterium* strain M. It has been shown here that RNA from the membrane fraction can take up labelled amino acids in an alkali-labile form, and this is probably an intermediate of protein synthesis as in the mammalian cell (Hoagland *et al.* 1958). On the other hand, we have not detected any appreciable binding of [^{14}C]amino acids to cytoplasmic RNA in tracer experiments with protoplasts, although the RNA from the membrane fraction in the same experiments has been labelled. Thus in strain KM at least the 1–2 % of RNA is probably an integral part of the membrane fraction. However, we have not obtained conclusive evidence that this bound amino acid can be transferred to the protein of the membranes. The rate of labelling of the RNA does not seem to be related in any way to the rate of labelling of the protein, but too little is at present known about the intermediate stages of protein synthesis to draw any definite conclusions. It has been shown (Cohn, 1959) that fractions of rat-liver microsomes can incorporate labelled amino acids efficiently into their protein fractions in the absence of detectable amounts of the pH 5·0 enzyme system, so that here, too, the role of RNA–amino acid complexes must remain open for the time being. It is unlikely that the cytoplasmic RNA of *B. megaterium* plays any direct part in protein synthesis. We have found (unpublished work) that prolonged centrifuging (20 hr. at 100 000 *g*) of cytoplasm derived from lysed protoplasts leads to the sedimentation of particles containing the bulk of the RNA, but tracer experiments with [^{14}C]amino acids have shown that these particles always contain protein of lower specific radioactivity than any other cellular fraction.

The results of the ultrasonic fractionation show that the labelling of the protein in the membrane fraction is far from uniform. At first it appeared that the solubilized fraction was the all-important one, especially when it was found that it contained the enzymes concerned with amino acid activation, and also amino acids bound to RNA. However, it was soon found that the isolated sedimentable fraction can incorporate labelled amino acids into protein whereas the 'soluble' fraction cannot do so (Table 3). This finding, coupled with the fact that short-term labelling experiments on whole membranes give [^{14}C]protein distributed equally between the two fractions, would seem to indicate that both fractions are necessary for full activity of the system. The small activity shown by the sedimentable fraction in isolation may well depend upon the presence of traces of the 'soluble' fraction for the preliminary activation of added amino acids.

Finally, evidence has been given that a further factor or factors may play a role in the incorporation process, and these factors appear to be associated with a lipid fraction. In contrast with results obtained in the rat-liver-microsome system (Littlefield, Keller, Gross & Zamecnik, 1955), extraction of labelled membranes with deoxycholate solubilizes a 'lipoprotein' fraction containing protein of much higher specific radioactivity than the residual protein. It has not yet been determined whether this 'lipoprotein' fraction corresponds to the fraction of the labelled protein solubilized by treating the membranes with lipase; we have not had available lipase completely free from proteolytic enzymes. However, it seems unlikely that the results obtained could be attributed entirely to the latter action. Prolonged digestion of labelled membranes with chymotrypsin in fact failed to remove more than a small fraction of the [^{14}C]-protein. The material removed by lipase is no

longer precipitable by trichloroacetic acid; this also applies to the bulk of the labelled compounds extracted by boiling methanol. A further chromatographic examination of the methanol extracts is being made. These results make it seem likely that lipids play a part in protein synthesis, and indeed the cytoplasmic membrane of *B. megaterium* has a high lipid content (Weibull & Bergström, 1958). Hendler (1958) has produced similar strong evidence for the existence of lipid intermediates in protein synthesis in the hen-oviduct system. If both RNA and lipids are concerned in protein synthesis, then our results would indicate that the lipids are involved at a stage subsequent to the action of RNA.

SUMMARY

1. A study has been made of the conditions necessary to obtain high and reproducible levels of incorporation of [^{14}C]amino acids into the protein fraction of the isolated cytoplasmic membrane of *Bacillus megaterium*.

2. Degradation of the labelled protein obtained has been carried out by partial and total acid hydrolysis. It was found that in all the cases investigated the added [^{14}C]amino acid had been incorporated unchanged into peptides.

3. Peptide-bond formation in the membrane system was inhibited by chloramphenicol to exactly the same extent as protein synthesis in the whole cell.

4. The cytoplasmic-membrane fraction contained amino acid-dependent enzymes catalysing the exchange of ^{32}P between [^{32}P]pyrophosphate and adenosine triphosphate. These enzymes are not inhibited by chloramphenicol.

5. The membrane fraction may be split into two fractions (soluble and sedimented) by treatment with ultrasonic vibrations. The soluble fraction contains the amino acid-activating enzymes and also the ribonucleic acid in an extractable form.

6. When the membranes are labelled with [^{14}C]-amino acids, the amount of radioactivity in the protein of the soluble fraction increases much more rapidly with time than that of the sedimented fraction. The ribonucleic acid of the soluble fraction carries bound radioactivity in a form not precipitable with hot trichloroacetic acid. It is readily removed by mild treatments at alkaline pH or by ribonuclease.

7. The possible role of the ribonucleic acid in the amino acid-incorporation process is discussed. Evidence has also been obtained indicating that lipids may play a part in protein synthesis.

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The Biochemistry of Aromatic Amines

6. THE METABOLISM OF 3:4-DIMETHYLANILINE IN RATS*

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3:4-Dimethylaniline is one of the simplest aromatic amines with carcinogenic properties. Morris, Lombard, Wagner & Weisburger (1957) showed that increases in the incidence of pituitary gland tumours occur when the amine is fed to rats, and Woolley (1953) and Woolley & Schaffner (1954) showed that the related *o*-aminophenol, 2-amino-4:5-dimethylphenol, causes a transient regression of spontaneous mammary tumours in mice. Allen, Boyland, Dukes, Horning & Watson (1957) showed that tumours are produced when pellets of the aminophenol and cholesterol are implanted in the bladders of mice. The metabolism of 3:4-dimethylaniline and of its *N*-acetyl derivative in rats is described below.

EXPERIMENTAL

Melting points are uncorrected.

Materials

3:4-Dimethylaniline had m.p. 53° and 3:4-dimethylacetanilide formed needles from benzene–light petroleum (b.p. 60–80°), m.p. 98°. *Potassium 3:4-dimethylphenyl sulphamate*, prepared by the action of chlorosulphonic acid in pyridine on 3:4-dimethylaniline (Boyland, Manson & Orr, 1957), separated from aq. ethanol in plates (Found: N, 6·0: $C_8H_{10}O_3NSK$ requires N, 5·85%).

Potassium 2-amino-4:5-dimethylphenyl sulphate was prepared by the action of alkaline $K_2S_2O_8$ on 3:4-dimethylaniline (Sims, 1958) and formed plates from aq. ethanol. 2-Amino-4:5-dimethylphenol was obtained from the sulphuric ester by hydrolysis with 6N-HCl and formed plates from light petroleum (b.p. 80–100°), m.p. 169–171° raised to 174–175° by sublimation at 150° and 0·5 mm. Hg. Diepolder (1909) gives m.p. 173–175° (turning brown at 165°). The aminophenol could be detected in aqueous solution at concentrations greater than 0·5 mg./ml. by shaking the solution with a little 2N-NaOH and ether, when a cherry-red ether layer separated.

2-Methyl-4-nitrobenzoic acid was prepared in poor yield by the oxidation of 3:4-dimethylnitrobenzene with HNO_3 as described by Jacobsen (1884). The nitro compound was reduced with hydrogen and Adams catalyst to yield 4-amino-2-methylbenzoic acid, which formed flat needles from water, m.p. 161–162° (decomp.). The acid was treated with acetic anhydride in 2N-NaOH, yielding 4-acetamido-2-methylbenzoic acid, which was purified by precipitation with 2N-HCl from a solution in aq. saturated $NaHCO_3$; it formed needles, m.p. 249° (decomp.). 4-Acetamido-2-methylbenzoic acid was also prepared by heating 3:4-dimethylacetanilide (2 g.) under reflux with water (200 ml.) whilst $KMnO_4$ (4 g.) was added during 10 min. The mixture was cooled and filtered and the filtrate was washed with ether (2 × 250 ml.), acidified to pH 4·0 with 2N-H_2SO_4 and extracted with ether for 6 hr. The product (950 mg.) obtained on evaporation of the ether had m.p. 228–240° (decomp.), which was raised, after repeated dissolution in aq. saturated $NaHCO_3$ and precipitation with 2N-HCl, to 249° (decomp.), undepressed in admixture with the product obtained by the first method. The acetamido compound was heated to 100° with an excess of 6N-HCl for 1 hr., when the product, extracted with ether after the addition of an excess of sodium acetate to the reaction mixture, formed flat needles from water, m.p. 162° (decomp.), undepressed in admixture with 4-amino-2-methylbenzoic acid prepared as described above.

Paper chromatography

Downward development on Whatman no. 1 paper was carried out with the solvents indicated in Table 1. The chromatograms were sprayed with either Ehrlich reagent (*p*-dimethylaminobenzaldehyde, 0·5% in ethanol containing 1 ml. of concn. HCl/100 ml.), a solution of *p*-dimethylaminocinnamaldehyde (2 g.) in 6N-HCl (100 ml.) and ethanol (100 ml.), as described by Harley-Mason & Archer (1958), or successively with aq. $NaNO_2$ (0·5%), 0·5N-HCl and 2-naphthol (0·5%) in ethanol–2N-NaOH (1:1, v/v). Other chromatograms were sprayed with 2N-HCl and heated in an oven to 70° for 15 min. before being sprayed with the above-mentioned reagents. The properties of the various metabolites on such paper chromatograms are listed in Table 1.

Isolation experiments

Eight rats (approx. 200 g.) were each given 3:4-dimethylaniline (50 mg.) in arachis oil (1 ml.) by forced feeding on alternate days until 7 g. of the amine had been administered. The urine was collected daily, pooled and stored at 0°. The preliminary treatment of the urine, the absorption of the aromatic compounds on activated charcoal and their subsequent elution was carried out as previously described (Boyland & Sims, 1958), except that the preliminary washing of the charcoal with aq. 2N-NH_3 soln. was omitted and the elution was carried out with methanol containing 5% (v/v) of aq. NH_3 soln. (sp.gr. 0·88). The material obtained on evaporation of the eluate was chromatographed on a column of cellulose powder (500 g., Whatman standard grade) with butanol–*cyclo*hexane–aq. 2N-NH_3 soln. (9:2:1, by vol.) as developing solvent. Fractions (200 ml.) were

* Part 5: Boyland & Manson (1958).

Table 1. *Properties on paper chromatograms of compounds related to 3:4-dimethylaniline*

Solvent systems and time of run: 1, butanol saturated with aq. 2N-NH_3 soln, 15 hr.; 2, butanol–propanol–water (2:1:1, by vol.), 15 hr.; 3, butanol–acetic acid–water (2:1:1, by vol.), 15 hr. Colours given in parentheses refer to those obtained after acid treatment of the paper chromatogram (see text). When the chromatograms were viewed under ultraviolet light all the compounds appeared as dark absorbent spots.

Compound	R_F in solvent 1	2	3	Ehrlich reagent	*p*-Dimethylamino-cinnamaldehyde reagent	Diazotization and coupling with 2-naphthol
3:4-Dimethylaniline	0·93	0·91	0·83	Yellow	Red	Pink
3:4-Dimethylacetanilide	0·88	0·88	0·92	Yellow* (yellow)	Red* (red)	—
2-Amino-4:5-dimethylphenol	0·82	0·78	0·84	Yellow turning brown	Red	Pale red
2-Amino-4:5-dimethylphenyl sulphate	0·42	0·51	0·65	Yellow turning brown	Red	Red turning brown
3:4-Dimethylphenyl sulphamate	0·37	0·45	0·59	Yellow* (yellow)	Red	Pink
4-Amino-2-methylbenzoic acid	0·11	0·86	0·87	Deep yellow	Violet	Pink
4-Acetamido-2-methylbenzoic acid	0·22	0·88	0·90	Yellow* (yellow)	Violet* (violet)	—
Conjugate, probably 4-amino-2-methylbenzoyl-glucosiduronate	0·07	0·20	0·53	Deep yellow	Violet	Pink

* Colour develops during 24 hr.

collected and separately evaporated to about 10 ml. under reduced pressure and the solutions were examined on paper chromatograms. The fractions were all tested for glucosiduronic acids with naphtharesorcinol: all tests were negative. The subsequent treatment of these solutions is described below.

Six rats were each given 3:4-dimethylacetanilide (75 mg.) in arachis oil (1 ml.) by intraperitoneal injection and the urines were collected for 24 hr. and treated as described below. Two pairs of rats were given 3:4-dimethylaniline (50 mg.) in arachis oil (1 ml.), either orally or intraperitoneally, and the urines, collected for 24 hr., were examined on paper chromatograms after a preliminary charcoal treatment as indicated above.

RESULTS

Fractions 1–3 from the cellulose column, which contained a little of two substances resembling 3:4-dimethylaniline and 3:4-dimethylacetanilide on paper chromatograms, were combined and evaporated; the residue was dissolved in 2N-H_2SO_4 and the solution extracted with ether (3 × 25 ml.) The ether was removed and the residue recrystallized from benzene–light petroleum (b.p. 60–80°) to yield 3:4-dimethylacetanilide (22 mg.), m.p. and mixed m.p. 95–97°.

Fraction 10 deposited a solid on standing which had m.p. 242° (decomp.), undepressed in admixture with 4-acetamido-2-methylbenzoic acid. Fractions 4–9 and the filtrate from fraction 10 were combined and evaporated, and the residue was dissolved in water (25 ml.) and extracted with ether for 4 hr. The ether-soluble material contained small amounts of compounds indistinguishable from 3:4-dimethylaniline and 4-acetamido-2-methylbenzoic acid on paper chromatograms. The pH of the aqueous layer was adjusted to 10 with 2N-KOH, the solution was evaporated to about 10 ml. under reduced pressure and an equal volume of ethanol was added. The crystals which separated overnight formed from aq. ethanol plates of potassium 2-amino-4:5-dimethylphenylsulphate (370 mg.) (Found: N, 5·35; S, 12·7. Calc. for $C_8H_{10}O_4NSK$: N, 5·5; S, 12·6%). The infrared spectrum was identical with that of the synthetic material. On hydrolysis with 2N-HCl at 100° the ester yielded 2-amino-4:5-dimethylphenol as plates from light petroleum (b.p. 80–100°), m.p. and mixed m.p. 169–171°. Paper chromatography showed that the mother liquors contained more of the sulphuric ester, together with a compound which was indistinguishable from 3:4-dimethylphenylsulphamic acid on all paper chromatograms.

Fraction no. 11 also deposited 4-acetamido-2-methylbenzoic acid, m.p. and mixed m.p. 241–243° (decomp.), on standing. The mother liquors from this fraction were combined with fractions 12–14 and evaporated to dryness and the residue was

warmed to 60° with 50 ml. of water. The solid was filtered off and the filtrate extracted with ether for 4 hr. The ether was evaporated, the residue was warmed with water (5 ml.) and the solid collected. The two solids were combined and purified by solution in aq. saturated $NaHCO_3$ and precipitation with 2 N-HCl to yield 4-acetamido-2-methylbenzoic acid in needles, m.p. and mixed m.p. 249° (decomp.) (Found: C, 62·0; H, 6·0; N, 7·3. Calc. for $C_{10}H_{11}O_3N$: C, 62·2; H, 5·7; N, 7·25%). The infrared spectrum was identical with that of the compound obtained by the permanganate oxidation of 3:4-dimethylacetanilide. A total of 2·37 g. of 4-acetamido-2-methylbenzoic acid was obtained from the various fractions. A sample of acetamido compound was hydrolysed with 6 N-HCl at 100° for 30 min. to yield 4-amino-2-methylbenzoic acid, m.p. and mixed m.p. 161–162° (decomp.). The mother liquors from fractions 11–14 were shown by paper chromatography to contain 2-amino-4:5-dimethylphenyl sulphate.

Fractions 15–19 were evaporated to dryness under reduced pressure, the residue was dissolved in water and the solution extracted with ether for 6 hr. Removal of the ether and recrystallization of the residue from water yielded 4-amino-2-methylbenzoic acid (152 mg.) in plates, m.p. and mixed m.p. 162–163° (decomp.).

Fractions 19–24 were found by paper chromatography to contain small amounts of 4-amino-2-methylbenzoic acid and traces of a substance which, for reasons presented below, is believed to be a conjugate of this acid with glucuronic acid. Further elution of the column did not yield any more metabolites.

The pooled urines from the rats given 3:4-dimethylacetanilide (450 mg.) were acidified with HCl and extracted with ether for 6 hr. Removal of the ether and purification of the residue by solution in aq. $NaHCO_3$ and precipitation with acid as described before yielded 4-acetamido-2-methylbenzoic acid (150 mg.), m.p. and mixed m.p. 247–249° (decomp.). The aqueous layer was treated with charcoal and the products obtained on elution of the charcoal were examined on paper chromatograms together with the products obtained from the rats dosed orally and intraperitoneally with 3:4-dimethylaniline. Examination of the chromatograms showed no qualitative and little quantitative difference in metabolism in the three cases. All the metabolites mentioned above were detected except 3:4-dimethylphenylsulphamic acid and 4-amino-2-methylbenzoic acid, whilst the substance believed to be a glucuronic acid conjugate of the latter acid was much more prominent. Evidence for the structure of this compound was obtained by eluting spots from paper chromatograms with methanol and hydrolysing the recovered materials with 2 N-HCl at 100° for 10 min. The hydrolysates, on paper chromatograms, contained 4-amino-2-methylbenzoic acid, but no glycine could be detected with ninhydrin. The unhydrolysed extracts gave positive naphtharesorcinol tests. A comparison on paper chromatograms of the compound detected in the urine with the product from the reaction of 4-amino-2-methylbenzoic acid with glucuronic acid showed that the compounds differed, the *N*-glucosiduronic acid being completely decomposed by solvent 3 and partially decomposed by solvent 2 (Table 1). It is probable therefore that the compound in the urine is 4-amino-2-methylbenzoylglucosiduronic acid.

DISCUSSION

There appears to be little difference in the metabolites of 3:4-dimethylaniline and 3:4-dimethylacetanilide, the metabolism of both compounds proceeding by two main routes. One involves an oxidation of the methyl group in the position *para* to the amino group, followed by either acetylation of the amino group or conjugation of the carboxyl group with glucuronic acid. The amino group may be acetylated before oxidation occurs, in which case conjugation of the carboxyl group with glucuronic acid must be accompanied by deacetylation. There was no evidence of conjugation of 4-acetamido-2-methylbenzoic acid with glucuronic acid. The second route involves a hydroxylation *ortho* to the amino group, followed by conjugation to give 2-amino-4:5-dimethylphenyl sulphate.

In the formation of 4-acetamido-2-methylbenzoic acid, the metabolism of the compounds resembles that of *p*-acetotoluidide, which Jaffe & Hilbert (1888) and Bray & Thorpe (1948) showed was converted by rabbits into *p*-acetamidobenzoic acid. Bray, Lake & Thorpe (1949) found that the methyl group of *N*-*p*-tolylurea is similarly oxidized. None of these workers, however, found evidence for hydroxylation and sulphuric acid conjugation, and Hildebrandt (1907) was unable to detect any sulphuric ester formation with *p*-toluidine in rabbits.

The absence of a glycine conjugate of 4-amino-2-methylbenzoic acid is not surprising: Quick (1932) found that substituents *ortho* to the carboxyl group inhibit glycine conjugation and Bray, Thorpe & Wood (1949) and Bray, Humphris & Thorpe (1949) found that little glycine conjugation occurs when *o*-toluic acid and *o*-xylene are given to rabbits. Bray, Lake, Neale, Thorpe & Wood (1948) isolated 4-aminobenzoylglucosiduronic acid from the urine of rabbits receiving *p*-aminobenzoic acid. Since 4-amino-2-methylbenzoic acid could not be detected in freshly voided urine, it is probable that the acid isolated by means of the cellulose column arose by the decomposition of the glucuronic acid conjugate.

There was no evidence for *N*- or phenolic *O*-conjugation with glucuronic acid; in this respect the results differ from those of Elson, Goulden & Warren (1958), who found that rats tend to excrete amines which have carcinogenic activity as glucuronic acid conjugates whereas non-carcinogenic amines are excreted as sulphuric esters. The presence of only small amounts of 3:4-dimethylphenylsulphamic acid is not unexpected since Boyland *et al.* (1957) found that rats usually excrete little of this type of metabolite after treatment with aromatic amines. The sulphamate was detectable only after repeated dosing with 3:4-dimethylaniline.

The two sites of biological oxidation (the methyl group attached to C-4 and the carbon atom at position 6) are the same as those in which chemical reactions occur with permanganate and persulphate respectively. There is no evidence of oxidation of the methyl group attached to C-3 or of hydroxylation at C-2 or C-5 in either the biological or chemical reactions. Bray & Thorpe (1948) found that with *m*-acetotoluidide, oxidation to the carboxylic acid occurred to a lesser extent than with *p*-acetotoluidide.

Which, if either, of the two metabolic routes is involved in carcinogenesis is not known, although 2-amino-4:5-dimethylphenyl sulphate, which is hydrolysed by a number of sulphatases (Boyland, Manson, Sims & Williams, 1956), could give rise to 2-amino-4:5-dimethylphenol (which is known to possess carcinogenic activity) on hydrolysis. The free aminophenol was not detected in the urine of rats treated with 3:4-dimethylaniline. Little is known of the carcinogenic activity of compounds such as 4-acetamido-2-methylbenzoic acid although simpler compounds such as *p*-toluidine are inactive. The results give no indication why 3:4-dimethylaniline is active mainly in the pituitary gland.

SUMMARY

1. 3:4-Dimethylaniline and 3:4-dimethylacetanilide are excreted by rats as 4-acetamido-2-methylbenzoic acid, 2-amino-4:5-dimethylphenyl sulphate and probably 4-amino-2-methylbenzoylglucosiduronic acid. Small amounts of 3:4-dimethylsulphamic acid and 3:4-dimethylacetanilide were formed from 3:4-dimethylaniline.

2. The behaviour on paper chromatograms of a number of these compounds is described.

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The Determination of Chlorine, Bromine and Iodine in Biological Material by Activation Analysis

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The determination of the halogens in biological samples is hampered by the fact that bromine and iodine are almost always present in trace amounts relative to chlorine. This is because of the relative abundance of these elements in the external environment. Thus in igneous rocks the Cl:Br and Cl:I ratios are about 200:1 and 1000:1 respectively, while in sea water they are 290:1 and 380 000:1 (Rankama & Sahama, 1950). While it is relatively easy to determine total halogen in any sample, the result refers almost entirely to the chlorine present, and special methods have to be employed to determine the bromine and iodine. Many halogen compounds are extremely volatile, and great care must be exercised to avoid losses in ashing biological material before determining these elements.

The method of activation analysis described in the present paper overcomes some of these difficulties. It is a fortunate coincidence that the cross-sections for thermal neutrons increase with atomic weight in the halogen series. This is clearly shown in Table 1.

This table shows that activation analysis for the halogens is capable of very great sensitivity, but it also illustrates the main difficulty of the method for these particular elements, which is the very short half-life of most of the nuclides. In particular, no attempt has been made to determine fluorine by this technique, since negligible radioactivity would be left in this element 2 min. after removal from the neutron source. The relatively long half-life of one of the 80 Br isomers gives plenty of time for chemical separation, but the chlorine and iodine must be separated with the least possible delay to avoid losses in sensitivity. Solvent-extraction methods have been used as far as possible, since these are far quicker than distillation or precipitation techniques.

One great advantage of the activation method is that no ashing need be attempted until the activation process is complete, after which inactive carrier can be added to determine losses in the subsequent chemistry, and contamination is minimized. The method has been tested on seeds and human blood.

Table 1. *Calculated induced radioactivities in halogens after exposure to a thermal neutron flux of* 10^{12} *neutrons/cm.*^{2}*/sec.*

Nuclide produced	Half-life	Radioactivity (disintegrations/min./10^{-9} g.) 30 min. exposure	4 hr. exposure
^{20}F	11 sec.	17	17
^{38}Cl	37 min.	62	130
^{80m}Br	4·6 hr.	49	180
^{80}Br	18 min.	140	2000
^{128}I	25 min.	980	1800

EXPERIMENTAL

Tomato plants were grown in sand culture and seeds were collected as described by Bowen (1959). Blood samples were collected from donors at a transfusion centre, in clean polythene beakers, from which weighed samples were transferred to polythene ampoules which could be sealed before activation. Samples of 1 ml. or more were partially dried before sealing. The standards, which were irradiated with the samples, consisted of 10 μg. of Cl, 0·2 μg. of Br and 1 μg. of I as the ammonium salts in solution. These were either sealed in polythene ampoules or absorbed on dry tomato seeds of known halogen content, a micropipette being used for delivery. Samples and standards were packed in a polythene bag inside a nylon or aluminium can for irradiation in the Harwell reactor B.E.P.O. in a flux of about 10^{12} thermal neutrons/cm.^{2}/sec. Activation times of 30 min. were used for chlorine and iodine determinations, and 4 hr. for bromine.

Chemical separation of chlorine. The activated sample (0·05 g. of seeds or 0·02 g. of blood) was transferred to a

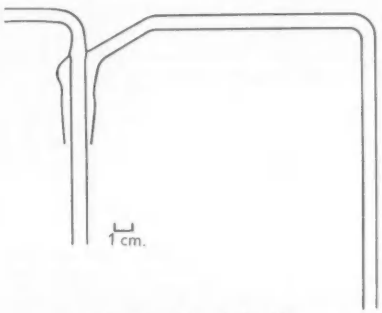

Fig. 1. Pyrex distillation head.

50 ml. round-bottomed flask containing 10 mg. of chlorine as ammonium chloride, 1 ml. of 90% phosphoric acid and 1 ml. of 16N-HNO_3. A distillation head (Fig. 1) was fitted, with a polytetrafluoroethylene sleeve between the cone and socket joint, and distillation was carried out for 5 min. at 150° in a slow current of air. The distillate was collected in 10 ml. of 5% sodium hydroxide solution in a 50 ml. separating funnel.

Where iodine and bromine contributed appreciably to the activity of the distillate, they were removed as follows: two drops of 5% ammonium iodide, 5 drops of 1% sodium nitrite and 5N-nitric acid were added to the funnel till the solution was acid. The solution was extracted three times with 10 ml. portions of carbon tetrachloride to remove iodine, the lower layer being discarded each time. Two drops of 5% ammonium bromide were then added and 2% potassium permanganate was run in drop by drop until its pink colour just persisted. Three further extractions with 10 ml. portions of carbon tetrachloride removed bromine. The aqueous layer was then made alkaline in a centrifuge tube and 5 drops of 10% ferric nitrate were added. The ferric hydroxide was spun off and the supernatant liquid was poured into a fresh centrifuge tube and made acid with nitric acid. Silver nitrate (3 ml. of 4% solution) was used to precipitate silver chloride, which was centrifuged, washed twice with water and once with acetone, and then transferred to an aluminium counting tray and dried. Counting was carried out with an end-window Geiger counter. The weight of the precipitate was used for calculating the chemical yield, and the count relative to that of the standard gave the amount of chlorine in the sample. Self-absorption corrections were negligible. Counting was repeated at 35 min. intervals to establish the radiochemical purity of the separated chlorine. The whole chemical procedure could be carried out in about 35 min. for two samples, or 1·5 hr. for eight samples.

Chemical separation of bromine. The activated sample (0·05 g. of seeds or 0·05 g. of blood) was distilled as described for chlorine, except that the 10 mg. of chlorine carrier was replaced by 20 mg. of bromine as ammonium bromide.

The distillate was made N with respect to nitric acid, and 2% potassium permanganate was added drop by drop until the pink colour just persisted. This solution was extracted three times with 10 ml. portions of carbon tetrachloride and the yellow non-aqueous layers were combined in a fresh separating funnel. They were shaken with 10 ml. of 0·5% sodium hydroxide until the yellow colour disappeared, and the lower layer was discarded. The aqueous layer was transferred to a 50 ml. centrifuge tube and made alkaline with sodium hydroxide, and five drops of 10% ferric nitrate solution were added. The ferric hydroxide was centrifuged and silver bromide precipitated in the same manner as described for the chloride. The separation time was about 1·5 hr. for eight samples.

The silver bromide was not counted until 6 hr. had elapsed after activation. In this time, the activity due to any contaminating halogens had decayed to negligible amounts and the most important activity left was that of ^{82}Br. Traces of the 36 hr. isotope ^{82}Br were also present, but it was unnecessary to correct for them. A small self-absorption correction was needed for precipitates of very different weights.

Chemical separation of iodine. The activated sample (0·5 g. of seeds or 1 g. of blood) was distilled as described above after 20 mg. of iodine had been added as ammonium iodide. If necessary a hot-air blower was used to complete the transfer of iodine to the receiving flask. It was not necessary to add a reducing agent to the sodium hydroxide solution to prevent formation of iodate.

The distillate was made N with respect to nitric acid, and bromide and chloride carriers were added. The iodine was twice extracted by shaking with sodium nitrite solution and carbon tetrachloride, and then re-extracted into 10 ml. of 0·5% sodium hydroxide in a fresh separating funnel. This extraction and re-extraction procedure was carried out twice for such samples as seeds with a relatively high iodine content, and three times for samples containing much chlorine. The final solution in aqueous sodium hydroxide was treated successively with two drops of 5% ammonium chloride, 1 ml. of aq. ammonia (sp.gr. 0·88) and 3 ml. of 4% silver nitrate. The precipitate was centrifuged and the supernatant liquid discarded. Washing was carried out with 2N-nitric acid, water (twice) and acetone, and the silver iodide was transferred to an aluminium dish and counted without delay. The whole chemical procedure could be carried out in 1 hr. for six samples.

Counting was repeated at 25 min. intervals to establish the radiochemical purity of the separated iodine. Samples were occasionally contaminated with ^{38}Cl. The self-absorption of ^{128}I β-particles was negligible.

Losses in the chemical steps were measured radiochemically on four replicates, and the mean chemical yields were 94% for Cl, 76% for Br and 85% for I. The greatest losses of bromine occurred in the permanganate oxidation step (Table 2). These losses are, of course, allowed for by measuring the chemical yield in each analysis.

Decontamination factors can be worked out from Table 2 for the separation of any individual halogen by any combination of chemical steps.

All analytical data reported below are the means of at least two replicates which agreed to within the estimated limits of accuracy of the technique (±5%).

Table 2. *Losses of halogens during separation*

The steps are described in detail in the text.

Chemical step	Loss (%)		
	Cl	Br	I
Distillation	2·4	0·5	3·3
CCl_4 extraction from aq. HNO_3	0·11	2·7	1·7
CCl_4 extraction from aq. $KMnO_4$	1·0	15·2	—
Precipitation of $Fe(OH)_3$	1·5	0·4	3·3
Silver halide ppt. in aq. HNO_3	1·4	1·3	5·6
Silver halide ppt. in aq. NH_3	99·59	7·3	3·2

RESULTS

Chlorine, bromine and iodine were determined in the seeds of tomato (*Lycopersicum esculentum*) and in the nutrient solution on which the tomato plants fed. The culture solution contained (μg./ml.) 3·67 Cl, 0·0104 Br and 0·00136 I, giving a Cl:Br ratio of 354:1 and a Cl:I ratio of 2700:1. Fresh seeds (moisture content standardized at 7·32%) contained (μg./g.) 66·7 Cl, 1·28 Br and 0·376 I, so that the respective ratios were 52·1:1 and 177:1. The results for blood with Br and I are shown in Table 3. For iodine, the contents in three other patients were (μg./g.) 0·0324, 0·0426, 0·0508; mean, 0·0419.

DISCUSSION

Errors in the activation method are different in kind from those normally encountered in analytical work. The neutron flux used for activation was homogeneous to $\pm 2\%$, and statistical counting errors were kept at about this level for the initial counts. There are a few nuclear processes other than (n, γ) reactions which might produce radiohalogens, e.g. the (n, p) reactions on the inert-gas isotopes ^{38}A, ^{80}Kr and ^{128}Xe. If samples containing considerable quantities of uranium were to be analysed by this method, allowance would have to be made for the short-lived fission products, e.g. ^{82}Br, ^{83}Br, ^{132}I, ^{134}I. No difficulty should arise with ordinary biological samples.

Errors whose magnitude is hard to assess could be caused by non-equilibration of active halogen and carrier, and by adsorption of halogen on the walls of the ampoule in which activation is carried out. Thus chlorine present in biological material as chlorate or perchlorate might not distil over during the wet-ashing process. The recovery figures quoted above, though satisfactory, give no information on this point. It is, however, widely assumed that virtually all the halogen in biological material is present either as inorganic or organic halides from which hydrogen halides are readily liberated by acid hydrolysis. Miller & Dodson (1950) have shown that energetic chlorine atoms produced by recoil after neutron capture undergo a Szilard–Chalmers reaction with solvent molecules or with the container walls. Activated halogens are also strongly adsorbed by polythene and polystyrene, but can easily be washed off Pyrex glassware. Every effort was made in the present work to wash all traces of the sample and standards into the distillation flask after activation.

Table 3. *Chlorine and bromine contents of blood of seven patients*

Patient	Cl (μg./g.)	Br (μg./g.)	Cl/Br ratio
A	3050	3·38	902
B	2890	1·85	1560
C	2800	1·66	1690
D	2720	1·56	1740
E	2590	3·95	657
F	2370	3·22	736
G	2700	3·65	740
Mean	2730	2·75	993

The formation of interhalogen compounds is not generally a serious difficulty in analytical work. Sometimes, when the determination of iodine and chlorine was being attempted in the same sample, all the iodine distilled over as iodine trichloride rather than as free iodine. This could be largely prevented by using more dilute nitric acid in the ashing mixture or by adding hypophosphite during the distillation.

Neutron-activation analysis has been applied to the halogens in a number of cases (e.g. Brues & Robertson, 1950; Atchison & Beamer, 1956; Cosgrove, Bastian & Morrison, 1958; for bibliography see Gibbons, Loveridge & Millett, 1957). It appears to be quite as sensitive and accurate as conventional methods for chlorine (cf. Johnson, Huston & Ozanne, 1958) and iodine (cf. Johnson & Butler, 1957; Rodgers & Poole, 1958). For bromine the technique is considerably more sensitive than any yet reported in the literature (cf. Ucko, 1936; Conway & Flood, 1936; Neufeld, 1936; Hunter, 1955).

There are few strictly comparable results for the halogen content of seeds, but the values found in the present work do not appear to be unreasonable. Johnson, Stout, Broyer & Carlton (1957) found 0·03 μg.atom of chlorine per tomato seed, corresponding to approximately 350 μg. of Cl/g. (dry wt.). The content of leaves is very much higher. Beeson (1941) has summarized published determinations of bromine in vegetables, which range from 1 to 14 μg./g., and the Chilean Iodine Educational Bureau (1952) list a large number of figures for iodine in tomatoes which range from 0·1 to 1·6 μg./g. (dry wt.). Hence only our chlorine figures fall outside the rather wide range of published results. This is not surprising as Johnson *et al.* (1957) analysed commercial seeds, while ours were specially grown on solutions containing very little chlorine.

It is therefore particularly interesting that the Cl/Br and Cl/I ratios should be so widely different in the seeds and in the nutrient solution which fed them. This phenomenon has long been known for marine algae, but has not previously been demonstrated for higher plants. The sand supporting the roots of the tomato plants was extensively purified with nitric acid and water washed before the experiment and is unlikely to have retained appreciable halide after this treatment. The plants were

grown in a closed greenhouse without filtered air, in a locality 100 miles from the ocean. It is difficult to conceive of any method by which extraneous bromide and iodide could enter these seeds other than by the normal process of translocation. The result suggests that bromine and iodine may be essential elements for the tomato plant, as has recently been demonstrated for chlorine (Broyer, Carlton, Johnson & Stout, 1954).

Recent work by Lehr, Wybenga & Rosanow (1958) indicates still more clearly that iodine is probably essential, but evidence for the essentiality of bromine is still very tenuous (Newton & Toth, 1952; Ulrich & Ohki, 1956; Ozanne, Woolley & Broyer, 1957).

The analytical figures for the halogens in blood give confidence in the reliability of the method, since they, too, agree with the rather wide range of published results. Thus Spector (1956) quotes 2500–3100 μg. of chlorine/g. and 0·03–0·13 μg. of iodine/g. as the range for normal human blood: these figures are based on a critical selection of a large number of published determinations. Most workers agree that the normal content of bromine in blood lies between 1 and 10 μg./g., with occasional high values for patients who have received bromides in medicine (Ucko, 1936; Conway & Flood, 1936). Our figures are somewhat lower than most others (e.g. Hunter, 1955), but agree with the determinations of Ucko (1936).

SUMMARY

1. Methods are described for the determination of chlorine, bromine and iodine in biological material by neutron-activation analysis. The ultimate limits of detection of the method are approximately 10^{-8} g. for chlorine, 10^{-9} g. for bromine and 10^{-10} g. for iodine.

2. The three halogens have been determined in seeds of the tomato (*Lycopersicum esculentum*) and in the nutrient solution on which the plant subsisted. Both bromine and iodine are concentrated relative to chlorine in the seeds, suggesting a physiological need for these elements.

3. The mean values for the three halogens in whole human blood were found to be 2730 μg./g. (Cl; 7 patients), 2·75 μg./g. (Br; 7 patients) and 0·0419 μg./g. (I; 3 patients).

4. The method is particularly recommended for the determination of bromine, since it is more sensitive and less subject to interference by other halogens than are other current methods.

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